Transient Binding, Oligomerization and Activity of the Antimicrobial Protein Granulysin on Eukaryotic and Prokaryotic Membranes

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SUMMARY

The focus of this study was to investigate how the cytotoxic protein granulysin, which is released from cytotoxic T lymphocytes and natural killer cells, interacts with eukaryotic versus prokaryotic membranes, how it is able to bind to and enter an infected cell, as well as how it can reach and kill the intracellular bacteria without harming the host cell.

Using surface plasmon resonance (SPR) and immunoblotting we found that granulysin binds to eukaryotic membranes using electrostatic interactions. The entering of granulysin through eukaryotic membranes can be explained by the reversible binding of granulysin on cholesterol enriched lipid raft microdomains on the host cell membrane. Granulysin is then taken up by the endocytotic pathway as we could show using confocal microscopy. The endocytotic compartments get more acidic in the later stages, promoting a pH dependent activity of granulysin, which we show by fluorescence release assays. Once granulysin reaches the phagosomes and lysosomes, where intracellular bacteria are located, it binds again and oligomerizes on the prokaryotic membranes, and finally permeabilizes the membrane, as we could show using atomic force microscopy (AFM). The oligomerization of granulysin was studied using fluorescence resonance energy transfer (FRET) techniques.

This finding is of importance for understanding how granulysin and other positively charged antimicrobial proteins bind to the host cell membrane, are endocytosed and then capable to bind again to the bacterial membrane, leading to lysis of the intracellular bacteria.

ZUSAMMENFASSUNG

Ziel der vorgeliegenden Arbeit war die Interaktion des, von zytotoxischen T-Zellen und natürlichen Killer Zellen freigesetzten, Protein Granulysin mit pro- und eukaryotischen Membranen zu analysieren. Darüber hinaus wurde untersucht wie Granulysin über die Membran einer infizierten Zelle aufgenommen wird und nachfolgend intrazelluläre Bakterien erreicht um diese zu töten, jedoch ohne die Wirtszelle zu schädigen.

Mit Oberflächen-Plasmon-Resonanz (SPR) und Immunoblotting konnten wir nachweisen, dass Granulysin durch elektrostatische Wechselwirkungen an eukaryotische Membranen bindet. Die Aufnahme von Granulysin durch die eukaryotische Membran kann durch eine reversible Bindung des Proteins an cholesterolhaltige Lipid Raft Mikrodomänen auf der Membranoberfläche erklärt werden. Durch konfokale Laserskanning Mikroskopie konnten wir nachweisen, dass Granulysin mittels Endozytose aufgenommen wird. Durch Fluorezenzfreisetzungstests konnten wir eine starke pH Abhängigkeit von Granulysin zeigen. Granulysin befindet sich nach der Aufnahme gelöst in endozytotischen Vesikeln, deren pH Wert im Laufe der Reifung abnimmt. Durch diesen pH Abnahmen wird die Bindungskapazität des Granulysins an Membran gefördert. Eine Verschmelzung der Granulysin beinhaltenden Endosomen mit Lysophagosomen, die intrazelluläre Bakterien enthalten können, führt zum Binden von Granulysin an die Membranen intrazellulären Prokaryoten. Da durch werden diese permeabilisiert, was wir mit Raster Kraft Mikroskopie (AFM) zeigen konnten. Es kommt hierbei zu einer Oligomerisierung der Granulysinmoleküle in der Prokaryotenmembran, was wir durch Fluoreszenz Resonanz Energietransfer (FRET) nachweisen konnten.

Möglicherweise lässt sich Granulysin also Modellprotein für andere positiv geladene antimikrobische Proteine verstehen, die einen ähnlichen Wirkungmechanismus aufweisen könnten. Ob der Mechanismus der endozytotischen Aufnahme mit nachfolgender

Oligomerisation in der Bakterienmembran universell für alle bakterienlysierenden Proteine gilt, werden nachfolgende Untersuchungen zeigen.

IMMUNE RESPONSE AS DEFENCE AGAINST INTRA-CELLULAR BACTERIAL INFECTIONS

Humans have several mechanisms as defence against infections. The first line of defence is the mechanical anatomical barrier of the skin and internal epithelial layers. The second line comprises two systems that act in cooperation as protection against infections: the cell mediated innate immune response, which rapidly recognizes preserved molecular patterns on pathogens and the cell mediated adaptive immune response that relies on gene rearrangement and clonal expansion of antigen-specific lymphocytes (Litman, Cannon & Dishaw, 2005; Perregaux et al., 2002). The immune defence against intracellular bacteria is mediated by antibodies and cytotoxic T lymphocytes (CTLs) of the adaptive immune response as well as natural killer (NK) cells of the innate immune response (Flynn et al., 1993; Krensky, 2000; Liu, Walsh & Young, 1995b). The main focus of this study is directed on the defence against intracellular infections and the mechanism by which bacteria are killed by the immune system.

Cell Mediated Immunity of the Innate Immune Response

The cell mediated immunity of the innate immune response acts in response against intracellular bacterial infection. The leukocytes of the innate system include the NK cells, which are crucial in the antibacterial defence mechanism. NK cells release cytotoxic proteins resulting in death of infected cells as well as intracellular bacteria. Other processes are induced e.g. by mast cells that rapidly release granules containing histamine, heparin and chemokines as well as chemotaktic cytokines into the environment leading to attraction of leukocytes to the site of infection (Finlay & McFadden, 2006). Cytokines, such as IL-12, contribute to the proliferation and activation of NK cells (de Bruin et al., 2005; Hafner et al., 1999; Shibakura et al., 2003).

Cytokines that activate the recruitment of leukocytes into tissue engage also the expression of endothelial adhesion molecules (E-selectin, P-selectin, ICAM-1 and VCAM-1) and specific chemoattractants such as chemokines (Bochner, 2000). The first step involves rolling of leukocytes on the endothelial surface, which is mainly mediated by the selectin family. Rolling is followed by adhesion, which is mediated by leukocyte β1 and β2 integrins binding to the adhesion molecules ICAM-1 and VCAM-1 (Bochner, 2000). After the migration through the endothelium, the chemokines in the tissue determine where the leukocytes will localize and finally lead to lysis and killing of the intracellular bacteria (Constantin et al., 2000).

Natural Killer Cells

Natural killer (NK) cells ($CD56^{+}$) are essential for the cellular aspect of the innate immunity. They respond to help in bacterial infections in an antigen-independent manner, before the development of the adaptive immune system (Harty, Tvinnereim & White, 2000; Smyth et al., 2005). NK cells are able to distinguish between infected and malignant cells from normal host cells using receptors that recognize specific major histocompatibility complex (MHC) class I molecules. NK receptors are either member of the immunoglobulin (Ig) superfamily or of the C-type lectin superfamily. The activation of these receptors gives an inhibitory signal that does not induce NK mediated killing. Infection, but also transformation leads to reduced levels of MCH class I molecules, resulting in a higher probability of NK cell activity and leads therefore to killing of the pathogen (Smyth et al., 2005)

NK cells kill malignant and infected host cells by releasing cytotoxic proteins, such as perforin, granzyme B and granulysin, from acidic granular compartments and also by binding of the receptors Fas and TRAIL-R on the target cell (Sato et al., 2001). This mechanism is similar to the killing mechanism of CTLs, and will be described later.

Cell-Mediated Immunity of the Adaptive Immune Response

The adaptive cell-mediated immunity is triggered by priming and activation of T cells by antigen presenting cells (APC). The maturation of the effective cell-mediated immune response requires a proper activation of the CTLs by APC in the secondary lymphoid organs, and also the migration of the responding T cells to the particular site of the antigen in the body. The effectiveness of the T cell activation in the lymphoid organs depends on the concentration of the peptide-antigen and also the affinity of the T cell receptor (TCR) towards the antigen/MHC complex (Deeths & Mescher, 1999).

The integrin family and their ligands are important for the migration and activation of the CTLs. The most important integrin for CTLs is LFA-1 (lymphocyte function associated molecule) and its ligands, the intercellular adhesion molecules ICAM-1, ICAM-2 and ICAM-3, which are particularly expressed on APCs. The antigen-independent binding between the APC and the CTL, which is assisted by LFA-1 and ICAMs, by increasing the capability of the T lymphocyte to detect antigenic complexes presented on APCs. The activation of the T cell through the TCR results in a conversion of the LFA-1 from a low-avidity state to a high-avidity state for the binding of ICAMs (Jenkinson, Williams & Morgan, 2005; Kanwar et al., 2003).

After activation and maturation, these T cells are antigen-specific CTLs and they release various cytokines in response to each antigen, such as IL-2, the major interleukin inducing clonal T cell proliferation (Kanoh et al., 2002; Liu et al., 2002). Upon recognition of antigens, T cell differentiation finally results in migration to the site of the bacterial infection. The cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells (Horowitz, Friedlaender & Qian, 1996; White et al., 2000).

Cytotoxic T Lymphocytes

The CTLs ($CD8^+$, $CD4^+$, NKT, γδ) are capable of inducing death of infected somatic or tumor cells and also damaged cells and to kill intracellular bacteria in infected cells (Renkvist et al., 2001; Tynan et al., 2005). Most cytotoxic T cells express αβ-T-cell receptors that can recognize a specific antigen bound to MHC class I molecule, present on all nucleated cells, and a glycoprotein called CD8 in $CD8^+$ T cells, which serves as a co-receptor specific for the MHC class I molecule (Hein et al., 1995; Iannacone et al., 2005; Malek, 2003). The T cell antigen receptor does not interact with native antigens, but it is specific for a form of the antigen that is presented in combination with the MHC class I molecule (Vitetta et al., 1989). The elimination of the infection using cellular mechanisms, is completed via production of cytokines (IFN-γ) or by cell mediated cytolysis releasing cytotoxic proteins (Kaspar et al., 2001; Keefe et al., 2005; Sun et al., 2004). The mechanism by which these cytotoxic proteins are mediating target cell death is not completely understood, but it involves that perforin, one or more of the five human granzymes and/or granulysin are released from the granules of CTLs and NK cells. Perforin molecules associate with the membrane of the target cell by binding through the mannose 6-phosphate receptor, and by forming membrane pores. Through there pores, granzymes and granulysin have been thought to be able to enter the infected cell (Kaspar et al., 2001; Liu, Persechini & Young, 1995a; Liu et al., 1995b; Smyth et al., 2001). A second way to induce apoptosis is via cell-surface interactions between the cytotoxic T lymphocyte and the infected cell. When a cytotoxic T lymphocyte is activated it begins expressing the surface protein Fas ligand, which is able to bind Fas molecules expressed on the infected target cell and activate a cascade-dependent apoptosis (Dalton et al., 2004; Zeytun et al., 1997).

Cytotoxic Response against Intracellular Pathogens

The cytotoxic proteins secreted from CTLs and NK cells contribute to the host defence against intracellular pathogens (Kaspar et al., 2001; Smyth et al., 2001). The granule-protein mediated response against bacterial infections has been studied on a number of intracellular bacteria, and one microorgansim of great interest is *Mycobacterium tuberculosis*. CTLs and NK cells have shown to be protective against *M. tuberculosis* infections (Gansert et al., 2003; Stegelmann et al., 2005; Stenger et al., 1998). The cytotoxic proteins are released from cytotoxic granules into the immunological synapse after binding of the antigen specific CTL or NK cell to the target cell. The cytotoxic proteins are rapidly secreted from the CTLs and NK cells because they are stored in preformed secretory organells, with lysosomal membrane proteins and proteolytic enzymes (Burkhardt et al., 1990). These proteins include perforin, granzymes and granulysin, which act either alone or in combination with each other (Kaufmann, 1999; Pena & Krensky, 1997). The expression of these cytotoxic proteins is highly restricted to activated CTLs and NK cells (Peters et al., 1989).

Regardless of that central effector function of these cytotoxic proteins, the killing mechanism and uptake into target cells have not been well investigated, but it is known that binding and clustering of perforin and granulysin at the infected target cell membrane leads to uptake and finally lysis of the intracellular bacteria (Anderson et al., 2003).

Perforin

Perforin is one of the major cytolytic proteins of cytolytic granules of $CD8^+$, γδ, NKT cells and $CD56^+$ cells. Shinkai et al. (1988) found that perforin is expressed only in killer cell lines and not in helper T cells (Shinkai, Takio & Okumura, 1988). It has been shown that mice lacking perforin have lowered protection against intracellular bacteria, which is caused by granzyme not being able to enter the cytosol of the target cell without perforin. Within the

group of proteins released from the cytotoxic granules, perforin is the killer cell delivery molecule (Kagi et al., 1996).

Perforin is synthesized as a 70 kDa inactive precursor, which is then cleaved at the C-terminus to achieve the active 60 kDa form. This processing occurs in the acidic granules of the CTLs and NK cells, with a pH of 5.5 (Kataoka et al., 1994). The cleavage of perforin to the active form takes place at the boundary of the C2 domain at the C-terminus of perforin. This C2 domain is then able to bind to the plasma membrane and to initiate pore-formation, due to the conformational change that occurs on the perforin monomer after the cleavage (Young et al., 1986). This conformational change takes place in the presence of calcium ions, which leads to the binding of perforin to the phosphatidylcholine groups of the membrane lipids and to oligomerize to form a pore (Keefe et al., 2005; Tschopp et al., 1989). The purpose of perforin was originally thought to be to the insertion into the target membrane for pore-formation to allow the uptake of other granule proteins and for them to be delivered to the target cell cytosol. But it seems that the formation of these perforin pores are not a prerequisite for the uptake of the other granule proteins; granzymes and granulysin (Kurschus et al., 2004).

Calreticulin, which is a calcium storage protein in the cytotoxic granules, colocalizes with perforin and both proteins are released together from the cytotoxic granules. Perforin interacts with the P-domain of the calreticulin molecule. This domain has a high affinity for binding $Ca2^{+}$. Perforin and calreticulin dissociates after release, when being exposed to higher extracellular $Ca2^{+}$ concentrations. For perforin, calreticulin performs as a $Ca2^{+}$ regulated chaperone, and serves to protect CTLs and NK cell during biogenesis of cytotoxic granules (Andrin et al., 1998). The perforin mediated cell death is induced either by uncontrolled influx of small molecules such as $Ca2^{+}$ from the extracellular fluids or by induction of osmotic stress resulting in colloid osmotic lysis of the target cell. High concentrations of perforin leads to target cell apoptosis (Liu et al., 1995a; Liu et al., 1989).

Granzymes

Granzymes are proteases belonging to the chymotrypsin superfamily. A large number of granzymes have been characterized in human (granzyme A, B, H , K and M) (Kaiserman et al., 2006). Granzyme A and granzyme B are most studied granzymes. Granzyme A is a specific tryptase, which is stored as an inactive monomer and acts as a 65 kDa homodimer. It becomes concentrated in the nucleus of target cells, and together with granzyme B it enhances DNA fragmentation. Granzyme A cleaves substrates after basic residues and induces caspase independent apoptosis (Trapani et al., 1998).

The 26 kDa serine proteinase granzyme B can in addition of induction of apoptosis by activation of caspases, also directly cleave cytoplasmic substrates such as the thrombin receptor and the actin-binding protein filamin (Browne et al., 2000). Granzyme B is crucial for the rapid induction of target cell apoptosis by CTLs. Perforin can greatly enhance the uptake of granzyme B (Jans et al., 1996; Shi et al., 2005), but this protein can also enter infected cells in a perforin-independent manner (Froelich et al., 1996). Even though granzymes are endocytosed in a perforin independent manner, perforin is needed for the intracellular delivery of granzymes. It is known that granzyme B alone is not effective in the target cell killing, but in combination with perforin, granzyme B is able to induce apoptosis of the target cell (Giesubel et al., 2006; Kurschus et al., 2004).

After entering the target cells, granzymes promote DNA degradation by cleaving histones, which leads to an easier access of deoxyribonucleases to the nucleus. However, it has been shown that granzymes are also indirectly involved in DNA degradation by either binding to nuclear proteins such as nucleolin, and then subsequently cleaving DNA (Smyth & Trapani, 1995). The entering of granzyme B into the cell in a perforin independent manner predicts the existence of a cell surface receptor for granzyme B. Motyka et al. (2000) presented verification that this receptor is the cation-independent mannose 6-phosphate receptor called

IGF2R. Inhibition of the interaction between granzyme B and the IGF2R receptor prevents granzyme B from binding to the cell surface, which subsequently prevents uptake, and the induction of apoptosis. Significantly, expression of the IGF2R receptor is necessary for CTL-mediated apoptosis of target cells in vitro (Motyka et al., 2000; Patel, Gores & Kaufmann, 1996). Granzyme also enters the cell by binding to the cell surface due to the positive charges on the protein. It has been shown that the binding of granzyme B is mainly dependent on the charges (Shi et al., 2005).

THE ANTIMICROBIAL PROTEIN GRANULYSIN

Granulysin was first mentioned by Jongstra el al. (1987), who found a gene encoding for the protein 519, which is expressed late after T cell activation. Pena et al. (1997) described 519 as a lytic molecules stored in and released from the cytotoxic granules of CTLs and NK cells called granulysin (Jongstra et al., 1987; Pena et al., 1997).

The antimicrobial protein granulysin is active against a broad range of pathogens, such as *Listeria monocytogenes, Mycobacterium tuberculosis* and *Trypanosoma cruzi* (Clayberger & Krensky, 2003; Krensky, 2000; Stenger et al., 1998). One detectable effect of the action of granulysin against *M. tuberculosis* is the development of protruding lesions on the target cell surface (Stenger et al., 1998), which increases the permeability of the membranes resulting in osmotic lysis. Together with perforin, granulysin is able to enter the infected host cell and to kill intracellular bacteria without simultaneous apoptosis. Though, the structure of granulysin suggests that it could be able to bind to and perturb membranes also in a perforin-independent manner (Anderson et al., 2003; Thoma-Uszynski, Stenger & Modlin, 2000).

Processing, Regulation and Activation of Granulysin

The gene of granulysin is located on the human chromosome 2 (Donlon, Krensky & Clayberger, 1990) and includes six exons within a 3,9 kb genomic locus encoding at least four alternative spliced transcripts (NKG5, 519, 520 and 522), all differing mainly in exon 2. The most abundant transcript in the functional human CTL cell line AJY was 520 appearing late after T cell activation (Manning et al., 1992). Since the transcript 520 has a fitting Kozak sequence for the beginning of translation, this transcript was thought to be responsible for the large amount of granulysin produced (Pena & Krensky, 1997). NKG5, a similar transcript to 520, was not found in the AJY, but would have the same purpose in NK cells (Houchins et al.,

1993). We also found in primary cultured cells the transcripts for NKG5, two variants of 519 and two novel short forms of granulysin (Latinovic-Golic et al., 2007).

Granulysin is expressed as a 15 kDa protein and then processed by proteolytic cleavage at both N and C termini of the 15 kDa precursor to the active 9 kDa form (Hanson et al., 1999). The 15 kDa form of granulysin is rapidly produced, but has a shorter half-life and is poorly secreted, whereas the secretory 9 kDa form is produced slowly and is relatively stable (Pena & Krensky, 1997).

Structure of Granulysin and Related Proteins

Granulysin belongs to the saposin-like protein (SAPLIP) family, which includes amoebapores (Bruhn & Leippe, 1999), NK-lysin (Liepinsh et al., 1997) and saposin A, B, C and D (Clayberger & Krensky, 2003; Krensky, 2000; Morimoto et al., 1988; Morimoto et al., 1989; O'Brien & Kishimoto, 1991; Qi & Grabowski, 2001). The family members are cationic membrane interacting proteins and they share a particular polypeptide motive of a five-helical bundle and highly conserved cysteine residues that form disulphide bonds (Munford, Sheppard & O'Hara, 1995), which give the molecule a stable structure. They interact with a variety of lipids, especially negatively charged phospholipids, cholesterol and sphingolipids (Vaccaro et al., 1995; Vaccaro et al., 1999). Although the SAPLIP family members have structural similarities, they have various biological functions: amoebapores are capable of forming ion channels or pores in lipid membranes (Lynch, Rosenberg & Gitler, 1982; Young et al., 1982), saposin A - D modify the membrane to become substrates for enzymes, whereas granulysin and NK-lysin appear to directly permeabilize bacterial membranes (Anderson et al., 2003; Krensky, 2000).

Comparing the amino acid sequences of the SAPLIP family members reveals that granulysin has the highest identity to NK-lysin (43% identity), a porcine protein with antibacterial activity (Andersson et al., 1995). The structural comparision of NK-lysin and saposin C with

granulysin can be seen in Figure 1. NK-lysin is a 9 kDa cationic protein produced in cytolytic lymphocytes (Andersson et al., 1996; Andersson et al., 1995; Andreu et al., 1999; Stenger et al., 1998). It is expressed in $CD2^+$, $CD4^+$ and $CD8^+$ cells, which suggests that it functions as an effector protein of CTLs and NK cells. The structure consists of five amphipathic α-helices folding into a single globular domain with a hydrophobic core and a hydrophilic surface (Andersson et al., 1995). The charge distribution in NK-lysin implies a specific orientation for its primary contact to a negatively charged lipid bilayer, but similar to granulysin, the structure of NK-lysin does not suggest an obvious mechanism for pore formation (Liepinsh et al., 1997). It lyses membranes by molecular electroporation by burying one tryptophan residue (Trp58) in the bacterial membrane without changing its secondary structure, which results in pore formation or defects in the bacterial membrane (Miteva et al., 1999).

Saposin A, B, C and D are derived from the proteolytic processing of the precursor protein called prosaposin, and processed into four individual saposins and they also share the particular polypeptide motive of five α-helices and highly conserved cysteine residues known for the SAPLIP family members (Kolter & Sandhoff, 2005). Each individual saposin activates the breakdown of specific lipid substrates by assisting the access of the lipid headgroups to the active sites of cognate hydrolases. In the absence of these sphingolipid activators, the oligosaccharide chains of the membranelipids cannot reach far enough into the lysosomal lumen to be accessible to the active sites of the hydrolases. All saposins are able to activate lipid hydrolysis by solubilizing the lipid substrates or by destabilizing the membrane structure (Salvioli et al., 2000). Saposin C, which has the highest homology of all the saposins to granulysin, plays an important role in the activation of glucosylceramidase, the enzyme that degrades glucosylceramide and glucose in lysosomes (O'Brien & Kishimoto, 1991; Qi & Grabowski, 2001; Vaccaro et al., 1999).

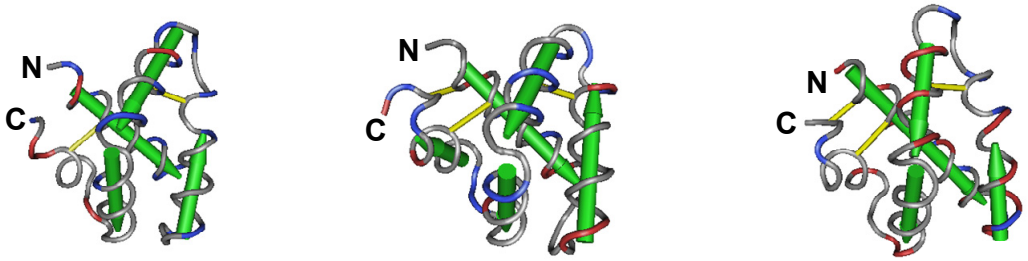

Figure 1. From left: schematic drawings of the three-dimensional structures of granulysin, NK-lysin and saposin C showing the charge distribution, the disulphide bonds and the helices. All these proteins are able to associate and modulate membranes, and they belong to SAPLIP family of lipid binding proteins.

Binding of Granulysin to Membranes

Granulysin is a highly positively charged molecule, which allows it to bind to the negatively charged bacterial membrane, but the structure of granulysin does not predict a pore-formation, suggesting another mechanism of action for perturbing the eukaryotic membrane and to lyse the intracellular prokaryotic membranes (Anderson et al., 2003). Granulysin, like to other members of the SAPLIP family, binds to the negatively charged bacterial or cell membrane. The crystal structure revealed by Anderson et al. (2003) suggests that the initial contact between granulysin and the membrane is mediated by the positive charged from the arginine residues attracted to negative charges in the membrane phosphates. Positive charges not involved in first contact with the membrane would maintain force to drive granulysin into the membrane surface, and could drive subsequent events, such as lysis of intracellular bacteria (Anderson et al., 2003).

Prokaryotic and Eukaryotic Membranes

One of the goals in this study was to find if granulysin is able to distinguish between prokaryotic and eukaryotic cells, which differ in the lipid composition of their membranes, and how granulysin may reach its intracellular target. An eukaryotic cell membrane consists of cholesterol, sphingomyelin and phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG), whereas the membrane of a prokaryotic cell lacks cholesterol and sphingomyelin, consisting mainly of cardiolipin, PC, PE, PS and PG, from which cardiolipin, PC, PE and PG are found in both membrane leaflets, whereas phosphatidylserine is mainly located in the cytoplasmic leaflet (Gidalevitz et al., 2003; Kurz et al., 2005; Pomorski et al., 2004; Tannert et al., 2003).

Eukaryotic cells are able to take up extracellular material, which are too large to be taken up via membrane pores, through the endocytosis pathway. These pathways are also utilized by bacteria and viruses. One endocytic mechanism is the receptor-mediated endocytosis via clathrin-coated pits (Husain & Moss, 2005; Orci et al., 1989). Another entry route into eukaryotic cells is through parts of the membrane driven by a coat made of caveolin, named caveolae (Nunes-Correia et al., 2004; Rothberg et al., 1992).

Additionally to clathrin-coated pits and caveolae, lipid rafts are also important mediators for endocytosis (Brown & London, 2000; Cambi et al., 2004; Manes, del Real & Martinez, 2003). They are specialized cholesterol-enriched microdomain in the eukaryotic membrane. Since 1972, it has been believed that, in cell membranes, phospholipids and membrane proteins are distributed all over according to a fluid mosaic model (Helms & Zurzolo, 2004; Singer & Nicolson, 1972; Somerharju, Virtanen & Cheng, 1999). However, in 1988, Kai Simons and Gerrit van Meer suggested the new idea that there exist microdomains enriched with cholesterol and sphingolipids, which are present in cell membranes (Simons & van Meer, 1988). This was the first time these microdomains were called lipid rafts. The original idea of

rafts was used as an explanation for the transport of cholesterol from the trans-Golgi to the plasma membrane (Simons & Ikonen, 1997).

Cholesterol has an important role in lipid rafts, but also in lipid bilayers, which usually exist in an ordered gel phase under the transition temperature of their lipids. However, cholesterol is able to eradicate the sharp transition between ordered gel phase and liquid crystalline phase, resulting in a liquid ordered phase, where the lipids have a high degree of lateral mobility as well as tightly packed acyl chains, illustrated in figure 2 (Brown & London, 2000; McMullen & McElhaney, 1997; Pralle et al., 2000; Simons & Ikonen, 1997). The difference in the eukaryotic and prokaryotic membranes, such as the lack of cholesterol in prokaryotic membranes, may be of importance for the mechanism of action of granulysin.

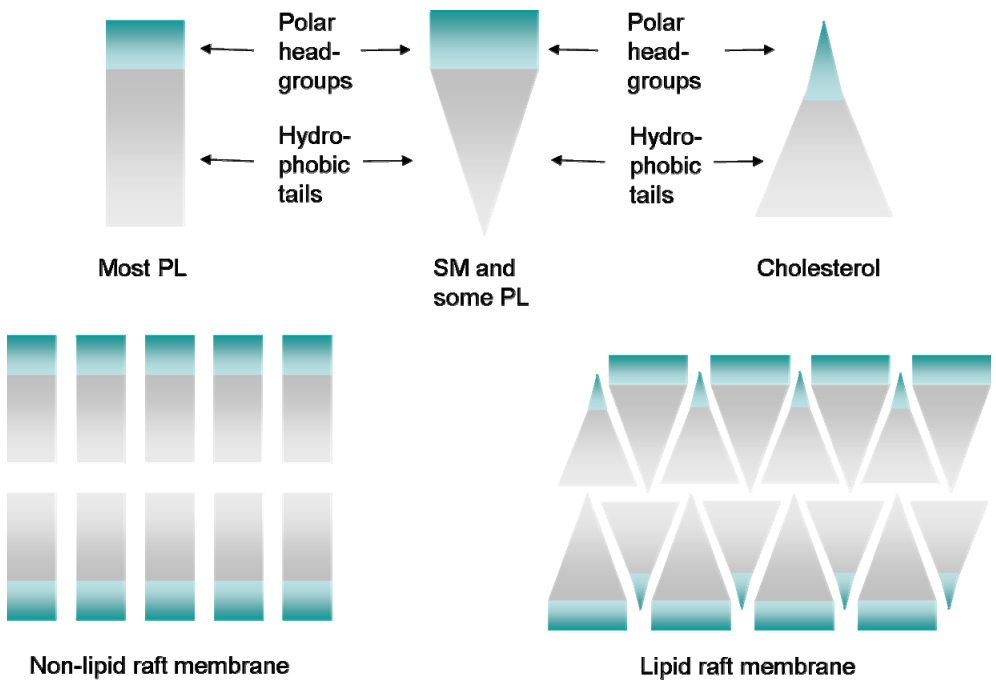

Figure 2. Lipid organization in membranes and lipid rafts. A schematic drawing based on the theoretical shape of phospholipids (PL), sphingomyelin (SM) and cholesterol showing the tighter packing of the cholesterol enriched microdomains.

OLIGOMERIZATION OF GRANULYSIN

Other cytotoxic proteins, such as streptolysin O, anthrolysin O and perfringolysin O, have been shown to form oligomers to be able to be active (Cocklin et al., 2006; Ramachandran, Tweten & Johnson, 2004; Ramachandran, Tweten & Johnson, 2005; Walev et al., 1995). These toxins first bind in a monomer form to lipid bilayers, then the molecules collide with one another, leading to a formation of non-covalently bonded polymeric aggregates (Alving et al., 1979; Cocklin et al., 2006; Johnson, Geoffroy & Alouf, 1980; Walev et al., 1995).

Also pores formed by perforin molecules grow in diameter through the progressive recruitment of additional monomers. Within these resulting oligomers of perforin molecules, the hydrophobic amino acid residues of the monomers face the acyl chains of the membrane lipids, whereas the hydrophobic residues are thought to line the interior of the oligomer. This barrel stave model also offers a molecular basis for the heterogeneity in size observed for perforin pores, which internal diameters ranges from 5 to 20 nm (Young & Cohn, 1986).

At this day, not much is known about the oligomerization of granulysin. It is known that the first contact is mediated by the positive changes of the arginine residues, but since the structure of the protein does not predict a pore formation, similarly to other SAPLIP family members (Anderson et al., 2003), and the size of the protein is too small to be able to independently cause lesions, the membrane permeabilization of bacterial membranes after binding of granulysin is thought to originate from oligomerization of the protein. The lesions shown by Stenger et al. (1998) on the membranes of *M. tuberculosis* are much larger than individual granulysin molecules, supporting the theory of oligomerization. Several granulysin molecules may participate in each membrane defect (Stenger et al., 1998). Anderson et al. propose a model for the mechanism of action for granulysin, where each granulysin molecule binds to its neighbouring molecules, applying local forces to a part of the membrane. This tight packing of the granulysin molecules could allow a cooperative bacterial membrane lysis

by a friction between the neighbouring molecules (Anderson et al., 2003). This model correlates with the carpet model, where protein molecules covers the infected cell by first binding to the membrane using electrostatic interactions and, second, permeabilization of the membrane is induced only where the protein concentration is high enough (Anderson et al., 2003; Pouny et al., 1992; Shai, 1999). However, an experimental basis has been missing for the mechanism of granulysin, that allows concise conclusions about the pore or defect formation of granulysin on eukaryotic and prokaryotic membranes.

AIMS OF THE STUDY

The central focus of this study was to investigate how the cytotoxic protein granulysin interacts with eukaryotic and prokaryotic membranes, as well as the mechanism how it enters the infected target cell to kill intracellular bacteria.

Aim 1:

The first aim was to show how granulysin is taken up by *Listeria innocua* infected host cells.

Aim 2:

The second aim was to find out how granulysin interacts with prokaryotic and eukaryotic membranes, as well as if the binding and permeabilization of eukaryotic membranes is different to prokaryotic membranes.

Aim 3:

The third aim was to find out the reason behind the possible difference in the interaction between granulysin and prokaryotic versus eukaryotic membranes and to study and visualize the binding and permeabilization of membranes with different charges and lipid components.

Aim 4:

The fourth aim was to investigate how granulysin is able to permeabilize membranes as well as the reason why eukaryotic cells are not lysed by granulysin.

RESULTS

Paper 1: Uptake of Granulysin via Lipid Rafts leads to Lysis of Intracellular *Listeria innocua*

Uptake of Granulysin via Lipid Rafts Leads to Lysis of Intracellular *Listeria innocua*

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The bacteriolytic activity of CTL is mediated by granulysin, which has been reported to kill intracellular *Mycobacterium tuberculosis* in dendritic cells (DC) with high efficiency. Despite that crucial effector function, the killing mechanism and uptake of granulysin into target cells have not been well investigated. To this end we analyzed granulysin binding, uptake, and the subsequent lysis of intracellular *Listeria innocua* in human DC. Recombinant granulysin was found to be actively taken up by DC into early endosomal Ag 1-labeled endosomes, as detected by immunofluorescence. Further transfer to *L. innocua*-containing phagosomes was indicated by colocalization of bacterial DNA with granulysin. After uptake of granulysin by DC, lysis of *L. innocua* was found in a dose-dependent manner. Uptake as well as lysis of *Listeria* were inhibited after blocking endocytosis by lowering the temperature and by cholesterol depletion of DC. Colocalization of granulysin with cholera toxin during uptake showed binding to and internalization via lipid rafts. In contrast to cholera toxin, which was targeted to the perinuclear compartment, granulysin was found exclusively in endosomal-phagosomal vesicles. Lipid raft microdomains, enriched in the immunological synapse, may thus enhance uptake and transfer of granulysin into bacterial infected host cells. *The Journal of Immunology,* **2005, 174: 4220–4227.**

Cytotoxic T lymphocytes and NK cells play an essential role in the host defense against intracellular pathogens such as *Chlamydia*, *Listeria*, and *Mycobacteria* (1). The mechanisms of CTL and NK cells involved in clearance of intracellular bacteria are release of cytokines (2), especially of IFN-γ and TNF-α, induction of target cell apoptosis (3), and direct mediation of antibacterial activity (4). The bacteriolytic activity of CTL is mediated by granulysin, a 9-kDa protein stored in cytolytic granules together with perforin and granzyme B (5). It was discovered by a subtractive hybridization procedure of late activated T cells (6, 7) and exhibited a vast spectrum of antimicrobial activity against bacteria, fungi, and parasites (8), either as free microorganisms or located in host cells. The killing of intracellular *Mycobacterium tuberculosis* by Vγ9/Vδ2 T lymphocytes was shown to be dependent on granulysin (9). Furthermore, it was reported that Vγ9/Vδ2 T cells from children with tuberculosis have strongly reduced effector functions, indicated by decreased IFN-γ production and granulysin expression, which was recovered upon chemotherapy (10). Other groups found granulysin responsible for the antimycobacterial activity of NKT cells (11) or CTL (8). Comparable results were obtained when investigating *Mycobacterium leprae*, which also survives within phagosomes of host cells (12, 13). Ochoa et al. (12) showed, by phenotyping of cells in dermal granulomas of leprosy lesions, that there are masses of granulysin-containing cells. These cells were identified as $CD4^+$ T cells. Moreover, the frequency of T cells containing granulysin in lesions reflected the capacity of the patients to restrict the disease. A recent study revealed granulysin-containing $CD4^+$ T cells infiltrating affected follicles and perilesional dermis in superficial microbial folliculitis (13). Together, these results indicate that there is little doubt that granulysin is crucial for the mediation of antibacterial activity of CTL and NK cells.

Granulysin belongs to the saposin-like protein family (SAPLIP).[2] These proteins share a particular polypeptide motive and affinity to a variety of lipids, especially sphingolipids (14), as well as to cholesterol (15). The interaction of saposins with sphingolipids has been extensively investigated. Dependent on the pH value, all saposins were reported to bind negatively charged gangliosides (16, 17). Positive charges at neutral pH are crucial for lytic activity of granulysin against bacteria and negatively charged liposomes (18). After binding and clustering of granulysin at the bacterial membrane, deformation of the membrane might lead to bacteriolysis (19).

Although the lytic activity of granulysin against a wide spectrum of microorganisms has been well studied (8, 20, 21), few data are available about the interaction of granulysin with the host cell itself, in particular on binding, uptake, and intracellular trafficking. Binding of granulysin may be mediated by lipid rafts, which are specialized membrane microdomains composed of sphingolipids and cholesterol in the outer exoplasmic leaflet as well as phospholipids and cholesterol in the inner cytoplasmic leaflet (22). There is abundant evidence that rafts are involved in a variety of cellular functions, including endocytosis of pathogens (23–25) and bacterial endotoxins (26, 27), as well as in protein sorting and ligand-induced signal transduction (28).

With respect to granulysin uptake in infected host cells, it is under debate whether other lytic proteins secreted by CTL and NK cells, such as perforin, assist in internalization of granulysin in

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[2] Abbreviations used in this paper; SAPLIP, saposin-like protein family; BAD, 2,3-butanedione; CAH, citraconic anhydride; CLSM, confocal laser scanning microscopy; DAPI, 4,6-diamidine-2-phenyl-indol-dihydrochloride; DC, dendritic cell; EEA-1, early endosomal Ag 1; His-tag, hexahistidine tag; LAMP-1, lysosomal-associated membrane protein 1; MCD, β-methyl-cyclodextrin; TSB, tryptic soy broth.

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infected host cells. Recombinant granulysin killed intracellular located *M. tuberculosis* only if cells were incubated simultaneously with perforin (8). In contrast, the killing of intracellular *M. tuberculosis* by $CD4^+$ T cells and $CD8^+$ CTLs was independent of perforin (29). $CD4^+$ T cells that occurred in leprosy lesions were negative for perforin (12). Furthermore, it is known from studies with perforin knockout mice that perforin is not required for the early control of mycobacterial infection in mice (30). Perforin-independent granulysin uptake has also been shown to some extent in Jurkat cells, where homogeneous distribution of granulysin in the cytoplasm of cells was found after incubation with high concentrations of up to 50 μM granulysin (31).

In our study we analyzed binding, uptake, and intracellular trafficking of granulysin using human monocyte-derived dendritic cells (DC) as hosts harboring *Listeria innocua*. DC are professional APCs that are crucial for the induction of a cellular immune response. They possess several mechanisms to internalize macromolecules and pathogens for Ag processing and presentation to T cells (32). The mechanisms involved in uptake are receptor-mediated endocytosis via clathrin-coated pits, phagocytosis, macropinocytosis, and lipid rafts (25, 33). *L. innocua* is a Gram-positive, apathogenic bacterium ubiquitously distributed in our environment (34). Upon phagocytosis, it is transferred via endosomes to phagosomes, where it resides until transfer to and lysis in phagolysosomes (35). Uptake and trafficking of granulysin were correlated to established markers of the endocytic pathway as well as to cholera toxin to evaluate whether lipid rafts are involved in a perforin-independent mechanism of granulysin uptake and killing of intracellular *L. innocua*.

Materials and Methods

Production of recombinant granulysin and anti-granulysin Abs

Due to the postulated C-terminal posttranslational processing of native granulysin in CTLs (5), two recombinant granulysins of different lengths were cloned from cDNA that was reverse transcribed from total RNA extracted from human lymphokine-activated killer cells. Both constructs corresponding to NKG5 started with G 63 and ended with D 132, or L 145, respectively, for granulysin$_{132}$ or granulysin$_{145}$. A fragment of human β-actin identical in length with granulysin$_{132}$ was additionally reverse transcribed for use as a control protein, referred to as actin$_{frag}$. These inserts were cloned in pEt28a (Novagen), followed by a factor Xa cleavage site (IEGR/G) and a C-terminal hexahistidine tag (His-tag). Proteins were expressed in *Escherichia coli* BL21 (DE3) additionally transformed with the chloramphenicol-resistant plasmid, pRARE (Novagen) in Luria-Bertoni medium containing 50 μg/ml kanamycin, 34 μg/ml chloramphenicol, and 2% glucose (all from Sigma-Aldrich). Expression was induced with 1 mM isopropyl-β-D-thiogalactoside (Qbiogene). After lysis of bacteria by adding 1 mg/ml lysozyme, 1% Triton X-100, 50 μg/ml DNase, and 5 μg/ml RNase (all from Sigma-Aldrich), granulysin was purified via nickel affinity chromatography and further renatured according the protocol of Ernst et al. (20). Renatured recombinant granulysin was additionally purified using Sep-Pak Vac 6cc (1 g) C18 cartridges (Waters) and was eluted with 100% acetonitrile containing 0.1% trifluoroacetic acid. After lyophilization, the protein concentration was determined using the Bio-Rad protein assay. Protein purity was estimated by SDS-PAGE and N-terminal sequencing (University of Zurich), revealing the correct first four amino acids as GRDY.

For Ab production and cleavage of the C-terminal His-tag, the dialysate containing granulysin$_{145}$ was concentrated by lyophilization, and after rehydration, it was treated with factor Xa (Amersham Biosciences) for 2 h at 4°C to remove the His-tag. The efficiency of His-tag removal was assessed by Western blotting and was routinely >99%. After His-Tag cleavage, granulysin$_{145}$ was reverse phase purified as described above. Polyclonal anti-granulysin Abs were raised in guinea pig (Pineda).

Viability determination of L. innocua

Serial dilutions of *L. innocua*-loaded cells lysed by adding ice-cold water or suspension-treated *L. innocua* were spread on tryptic soy broth (TSB; Difco) agar plates. CFU were determined by counting colonies after overnight culture at 37°C, and specific lysis was calculated using the formula ((CFUs in buffer control − CFUs in test incubation)/CFUs in buffer control) × 100.

Alternatively, turbidimetry was used to study specific lysis of bacteria (36). Serial dilutions of cell lysates or treated *L. innocua* were incubated in 96-well plates (Nunc). Bacterial growth curves were monitored in a microplate reader (Spectra MAX 340; Molecular Devices) at OD_{600} with discontinuous shaking for 16 h at 37°C. Specific lysis was calculated by determining the time when the maximum population was reached in buffer controls ($OD_{Tmax\text{-}control}$). At this point, the OD value ($OD_{Tmax\text{-}Test}$) of a shifted growth curve was evaluated, and specific lysis was calculated using the formula $((OD_{Tmax\text{-}control} - OD_{min}) - (OD_{Tmax\text{-}test} - OD_{min})/OD_{Tmax\text{-}control} - OD_{min}) \times 100$. All OD values were corrected by subtraction of the baseline OD (OD_{min}).

Bacteriolytic activity of granulysin

Granulysin was incubated at various concentrations for 3 h at 4 or 37°C with 10^5/ml *L. innocua* in 0.01 M Trisma base (pH 8). Actin$_{frag}$ and buffer alone served as controls. After incubation, the viability of *L. innocua* was determined as described above. For some experiments, granulysin$_{132}$ was pretreated with 2,3-butanedione (BAD) or with citraconic anhydride (CAH; both from Sigma-Aldrich) in 10 mM sodium borate buffer for 2 h at room temperature. During incubation, the pH was controlled and adjusted between 8 and 9. Granulysin$_{132}$ incubated in borate buffer alone served as a control. Binding of BAD and CAH to granulysin was analyzed using electrospray mass spectrometry (University of Zurich; not shown).

To study binding of granulysin to *L. innocua*, 10^6/ml bacteria were incubated with granulysin or actin$_{frag}$ in a concentration of 2.5 μM for 15 min at 37 or 4°C, respectively. Bacteria were washed subsequently three times with ice-cold PBS and fixed with 1.5% paraformaldehyde in PBS containing 1% sucrose.

Isolation and culture of DC

Human DC were generated in vitro from blood-derived precursors as previously described (37). Briefly, human PBMC obtained from venous blood of healthy donors (Blood Bank SRK) were isolated by Ficoll-Paque (Pharmacia Biotech) density centrifugation. The PBMC were cultured in RPMI 1640 supplemented with penicillin/streptomycin (all from Invitrogen Life Technologies) and 10% heat-inactivated pooled human A serum (Blood Bank SRK) for 2 h. The adherent cells were cultured for 6 days in RPMI 1640 supplemented with penicillin/streptomycin, 5% heat-inactivated pooled human A serum (DC culture medium) with rhGM-CSF (50 ng/ml; Novartis), and human rIL-4 (100 U/ml; R&D Systems).

Challenge of DC with Listeria

L. innocua were propagated in TSB at 37°C overnight, diluted 10-fold, and further expanded to an OD_{600} of 0.5 corresponding to 5×10^7/ml viable bacteria. Bacteria were harvested by centrifugation and washed twice with PBS before opsonization in RPMI 1640 with 50% pooled heat-inactivated human A serum for 30 min at 37°C. Opsonized *Listeria* were washed in PBS and resuspended in RPMI 1640. DC were challenged for 1 h with a multiplicity of infection of 5. Subsequently, cultures were washed with PBS and incubated for 3 h in DC culture medium containing 25 μg/ml gentamicin (Sigma-Aldrich) to kill extracellular *L. innocua*. Cholesterol depletion of DC was achieved by addition of β-methyl-cyclodextrin (MCD; Sigma-Aldrich) in RPMI 1640 without human serum for 1 h at 37°C.

Granulysin treatment of DC

L. innocua-challenged or unchallenged DC were incubated with various concentrations of granulysin, actin$_{frag}$, or culture medium alone for the indicated times at 37 or at 4°C, subsequently washed twice with PBS, and either fixed with 1.5% paraformaldehyde in PBS containing 1% sucrose for immunofluorescence labeling or lysed by adding ice-cold sterile water for 30 min on ice for assessment of viability of *L. innocua* as described above. DC were also incubated with granulysin$_{132}$ that was pretreated with BAD or CAH at the indicated concentrations in 10 mM sodium borate buffer or sodium borate buffer alone as a control. After incubation with modified granulysin for 1 h at 4°C, the cells were washed twice with ice-cold PBS and lysed with PBS containing 0.5% Triton. The content of granulysin bound to DC membranes was determined by Western blot analysis. Samples were run on a 15% SDS-PAGE gel and blotted on transfer membranes (Immobilon-P; Millipore). Granulysin was detected using anti-granulysin Ab. As a reference, cellular actin was detected using an anti-actin mAb (AC15; Sigma-Aldrich). The granulysin content bound to DC was measured and calculated relative to cellular actin using Image-J software (National Institutes of Health).

Confocal laser scanning microscopy (CLSM)

Fixed DC were cytospun onto glass slides and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 1 min at room temperature. Unspecific binding was blocked with 0.1% BSA (Fluka) in PBS for 1 h room temperature. Recombinant His-tagged granulysin$_{132}$, granulysin$_{145}$ after His-tag removal, or His-tagged actin$_{frag}$ were detected with an anti-His mAb (1/1,000; Invitrogen Life Technologies) or with guinea pig anti-granulysin antiserum (1/10,000). Lysosomes were labeled with a lysosomal-associated membrane protein 1 (LAMP-1) mAb (1/50), early endosomes were labeled with a rabbit early endosomal Ag 1 (EEA-1) Ab (1/200; Affinity BioReagents), and CD55 was labeled with an anti-CD55 mAb (Accurate Chemical). Omitting the first Abs served as a control for specificity. For detection, the following Abs were used: FITC-conjugated goat anti-mouse or a goat anti-rabbit Ab, Cy3-labeled goat anti-guinea pig Ab (all from Kirkegaard & Perry Laboratories), or Texas Red-conjugated donkey anti-mouse Ab (Jackson ImmunoResearch Laboratories). All Abs were diluted in 0.1% BSA in PBS. DNA was labeled with 1 μg/ml 4,6-diamidine-2-phenyl-indol-dihydrochloride (DAPI; Roche) in PBS for 15 min at room temperature.

For co-uptake experiments *L. innocua*-challenged DC were simultaneously treated with granulysin or actin$_{frag}$ in combination with cholera toxin-FITC (10 μg/ml; Molecular Probes), dextran-FITC (1 mg/ml; m.w. = 40,000; Sigma-Aldrich), or transferrin-FITC (25 μg/ml; Molecular Probes) for various time periods. Alternatively, *L. innocua*-challenged DC were pulsed with granulysin and cholera toxin-FITC for 10 min on ice. Subsequently, cells were washed twice with ice-cold PBS and transferred to 37°C for the indicated chase periods before fixation and processing as described above.

Fluorescent-labeled specimens were examined using a confocal laser scanning microscope (CLSM SP1; Leica). Images were analyzed using the Imaris software package (Bitplane), and threshold levels for calculation of colocalization micrographs were selected above background signals. Images representing single sections through three-dimensional volume stacks are shown.

Results

Bacteriolytic activity of recombinant granulysin

The antibacterial activity of granulysin$_{132}$ and granulysin$_{145}$, respectively, was monitored by assessing the viability of *L. innocua* in suspension. His-tagged granulysin$_{132}$-treated *L. innocua* were found to have a lowered viability depending on the granulysin$_{132}$ concentration used for incubation (Fig. 1*A*). This activity was identical with the antibacterial activity of granulysin$_{145}$ without a C-terminal His-tag. At a concentration of 2.5 μM, >90% of the bacteria were killed. Significant bacteriolysis could still be measured at a concentration of 0.15 μM. Actin$_{frag}$, a His-tagged fragment of human β-actin, identical in length, expression, and purification, used as a control to granulysin, did not affect *L. innocua* viability.

To investigate the kinetics of granulysin-induced bacteriolysis, *L. innocua* in suspension were incubated with 2.5 μM granulysin$_{132}$ or granulysin$_{145}$ without His-tag, and lysis was stopped by adding TSB at the indicated time points. Growth inhibition by granulysin was monitored and calculated from *Listeria* growth curves. The onset of bacteriolysis occurred very rapidly, with >70% of the bacteria killed after 5 min of granulysin treatment at 37°C (Fig. 1*B*). Fifteen minutes of granulysin incubation at 37°C was sufficient to kill 90% of the bacteria. Lowering the temperature to 4°C during incubation decreased specific bacteriolysis to 55% after 15 min and to 70% after 45 min, but did not abolish lysis (Fig. 1*B*).

After incubation with 2.5 μM granulysin$_{132}$ for 15 min and staining with an anti-His mAb, *L. innocua* were coated by granulysin$_{132}$ (Fig. 1*C*). Granulysin$_{132}$ bound to bacteria was also detected after treatment at 4°C (data not shown). No binding of actin$_{frag}$ was detected after treating the *Listeria* with actin$_{frag}$ (Fig. 1*D*). According to these findings, granulysin binds and kills *L. innocua* highly efficiently within minutes at 37 or 4°C.

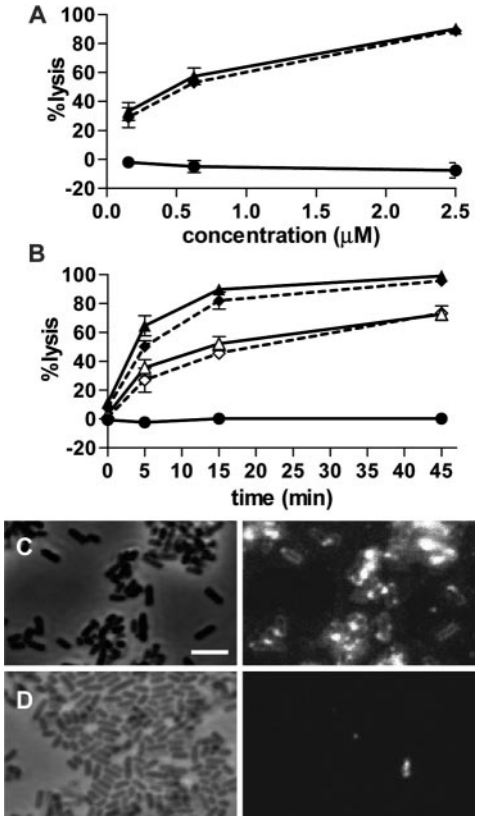

FIGURE 1. Bacteriolytic activity of recombinant granulysin against *L. innocua* in suspension. *A*, *L. innocua* were incubated for 3 h at 37°C with granulysin$_{132}$, granulysin$_{145}$, or actin$_{frag}$ as a control in various concentrations. Specific lysis was determined in CFU assays. The mean ± SE of three independent experiments are presented. *B*, *L. innocua* were treated with 2.5 μM granulysin$_{145}$, granulysin$_{132}$, or actin$_{frag}$ for various time periods at 37 and 4°C. Specific lysis was calculated from bacterial growth curves obtained by turbidimetry. The mean ± SE of three independent experiments are presented (▲, granulysin$_{132}$, 37°C; ◆, granulysin$_{145}$, 37°C; ●, actin$_{132}$, 37°C; △, granulysin$_{132}$, 4°C; ◇, granulysin$_{145}$, 4°C). Granulysin$_{132}$-treated (*C*) or actin$_{frag}$-treated (*D*) *L. innocua* were stained with an anti-His mAb for CLSM. Representative phase contrast (*left panels*) and immunofluorescence images (*right panels*) are shown. Bar = 4 μm.

Granulysin is actively taken up by DC

To lyse intracellular bacteria, granulysin has to enter the cell either by a passive or an active uptake mechanism. Active protein uptake is a temperature-dependent process; therefore, granulysin uptake was investigated at 37 and 4°C. For this purpose, DC were treated for 45 min at 37°C with granulysin$_{132}$ or actin$_{frag}$, and the localization of the proteins was determined by immunofluorescent staining using an anti-His mAb. CLSM revealed a spot-like pattern of immunolabeled granulysin$_{132}$ within the DC (Fig. 2*A*). Granulysin$_{132}$ distribution was identical in *L. innocua*-challenged DC (data not shown). After incubation for 45 min at 4°C, granulysin$_{132}$ was found exclusively at the cell membrane, and no significant transfer to an intracellular compartment occurred (Fig. 2*B*), indicating an active uptake mechanism. Actin$_{frag}$ was neither bound nor taken up by DC at detectable levels (Fig. 2*C*).

Ernst et al. (20) demonstrated that modification of arginine residues with BAD reduced the binding as well as the lytic capacity of granulysin against *E. coli*, whereas neither binding nor lytic capacity of granulysin pretreated with CAH to modify lysine residues was affected. Consistent with these results, pretreatment of granulysin$_{132}$ with BAD reduced the lytic activity against *L. innocua* in suspension, whereas CAH or borate buffer alone had no

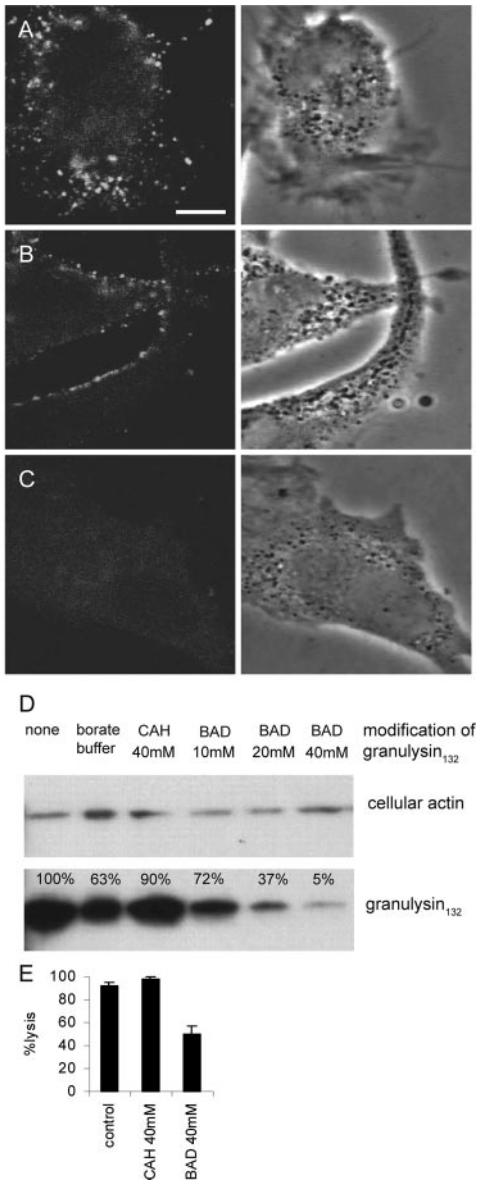

FIGURE 2. Granulysin$_{132}$ is actively taken up by human DC. Unchallenged DC were incubated for 45 min with 2.5 μM granulysin$_{132}$ at 37°C (*A*) or 4°C (*B*) or with 2.5 μM actin$_{frag}$ at 37°C (*C*). After the incubation, the cells were fixed and stained with the anti-His Ab for CLSM. Representative phase contrast (*right panels*) and immunofluorescence images (*left panels*) are shown. Bar = 8 μm. *D*, Granulysin$_{132}$ was pretreated with CAH, BAD, or sodium borate buffer before incubation with DC for 60 min at 4°C. The level of bound granulysin was assessed by Western blotting. Granulysin was detected with the anti-granulysin Ab. As a reference band, cellular actin was detected using an anti-actin mAb. *E*, The activity of modified granulysin$_{132}$ against *L. innocua* in suspension was tested with turbidimetry and calculated from bacterial growth curves.

effect (Fig. 2*E*). Preincubation of granulysin with BAD, but not with CAH, significantly decreased binding of granulysin$_{132}$ to DC cell membranes, as assessed by Western blotting (Fig. 2*D*). Similar to binding, uptake of granulysin$_{132}$ at 37°C was not affected by modification of lysine residues (not shown).

Granulysin binding and uptake in DC are associated with lipid rafts

The spot-like staining pattern of granulysin$_{132}$ within DC resembled vesicles of the endocytic pathway. Because granulysin belongs to the SAPLIP, which has known affinity to sphingolipids (5), a binding and initial uptake mechanism associated with lipid rafts seemed likely. Lipid rafts are highly organized microdomains in the plasma membrane with elevated cholesterol and glycosphingolipid contents (22). The β subunit of cholera toxin binds the G_{m1} ganglioside in lipid rafts and is a well-established marker to detect such microdomains (38). To examine possible association of granulysin binding and uptake in DC via lipid rafts, co-uptake experiments with fluorescently labeled cholera toxin and granulysin were performed. After incubation of granulysin$_{132}$ with cholera toxin for 30 min in steady state at 37°C, both proteins were found colocalized in DC (Fig. 3*A*, see colocalization panel). The colocalization of cholera toxin and granulysin$_{132}$ was most significant in the peripheral part of the cells. Cholera toxin was concentrated over time in the perinuclear area of the DC, whereas granulysin$_{132}$ remained in the peripheral compartment. No uptake of the proteins occurred at 4°C (Fig. 3*B*). Under these conditions granulysin$_{132}$ and cholera toxin were colocalized and remained in a patch-like pattern at the cell membrane. To exclude an interaction of the His-tag of granulysin$_{132}$ with the cell membrane, especially with lipid rafts, DC were coincubated with granulysin$_{145}$ after His-tag removal and cholera toxin at 37 and 4°C (Fig. 3, *C* and *D*). After fixation, the samples were stained with the anti-granulysin Ab. We found a staining pattern identical with that achieved with granulysin$_{132}$, proving that the His-tag did not influence granulysin binding or uptake in DC. The assumption of initial co-uptake and later separation of granulysin$_{132}$ and cholera toxin was confirmed in pulse-chase experiments. Granulysin$_{132}$ and cholera toxin were allowed to bind to DC on ice for 10 min, and subsequent to medium replacement, cells were incubated at 37°C for 5–90 min (Fig. 3*E*). After 5 min, both proteins were colocalized, either still bound to the cell membrane or in the peripheral compartments of the cells. Thirty minutes after binding to the cell membrane, granulysin$_{132}$ and cholera toxin had separated into different compartments. After 90 min, cholera toxin was found highly concentrated near the nucleus and was totally separated from granulysin$_{132}$, which remained in the peripheral compartment of the cells (see colocalization panels in Fig. 3*F*). The association of granulysin with lipid rafts in DC was confirmed by staining of granulysin$_{132}$-treated DC with an anti-CD55 Ab and an anti-granulysin Ab (Fig. 3*G*). To test possible involvement of macropinocytosis or clathrin-dependent endocytosis in granulysin$_{132}$ uptake, dextran-FITC and transferrin-FITC, respectively, were incubated with granulysin$_{132}$. In neither the initial uptake nor in the later stages was dextran (Fig. 4*C*) or transferrin (data not shown) colocalized with granulysin$_{132}$.

Preincubation of DC with MCD, which is known to disrupt lipid raft-mediated uptake by binding cholesterol (39), inhibited the uptake, but not the binding, of granulysin$_{132}$ in DC. After incubation of DC with MCD for 45 min at 37°C, granulysin$_{132}$ was detected in patches at the cell membrane (Fig. 4*A*). In contrast, simultaneously applied dextran-FITC was endocytosed by cholesterol-depleted cells at 37°C, indicating that the fluid phase endocytosis was still functional. After incubation of cholesterol-depleted cells at 4°C, both granulysin$_{132}$ and dextran-FITC were found at the cell membrane, but no intracellular staining was detected (Fig. 4*B*). In control cells pretreated with serum-free medium, uptake of both granulysin$_{132}$ and dextran-FITC was detected at 37°C (Fig. 4*C*). Similar results were obtained by pretreatment of DC with filipin (1 μg/ml), another lipid raft-disrupting drug (38), before granulysin$_{132}$ incubation (not shown). Overall, these findings revealed lipid rafts to be critically involved in initial uptake, but not binding of granulysin to DC.

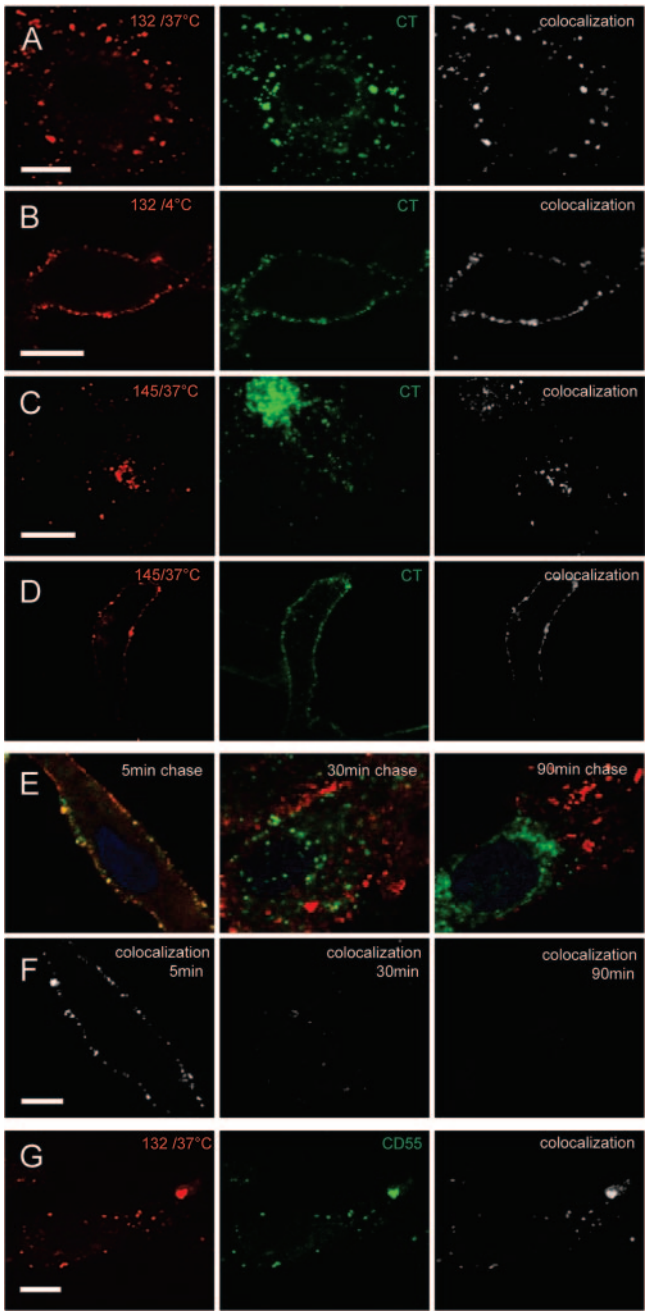

FIGURE 3. Granulysin binding and uptake in DC are associated with lipid rafts. *L.* innocua-challenged DC were coincubated with granulysin (2.5 μM; granulysin$_{132}$ (*A* and *B*) or granulysin$_{145}$ without His-tag (*C* and *D*)) and FITC-labeled cholera toxin (CT; 10 μg/ml) for 30 min at 37°C (*A* and *C*) or 4°C (*B* and *D*). *E* and *F*, For pulse-chase experiments, DC were pulsed with granulysin$_{132}$ and cholera toxin for 10 min at 4°C and subsequently cultivated at 37°C for the indicated chase periods. Granulysin$_{132}$ is marked in red, cholera toxin is shown in green, and nuclear and bacterial DNA are stained with DAPI (blue; merged image (*E*), calculated colocalization (*F*)). *G*, Double labeling of granulysin-treated DC with an anti-CD55 Ab and the anti-granulysin Ab. Granulysin$_{132}$ is marked in red, and CD55 is shown in green. Colocalization images of granulysin$_{132}$ and cholera toxin or CD55 were calculated using Imaris software. Bars = 8 μm.

Granulysin was found in early endosomes, but not in lysosomes

To follow trafficking of granulysin$_{132}$ from lipid rafts, double labeling of granulysin$_{132}$ with markers specific for the endocytic compartment was performed. In a first step, early endosomes were

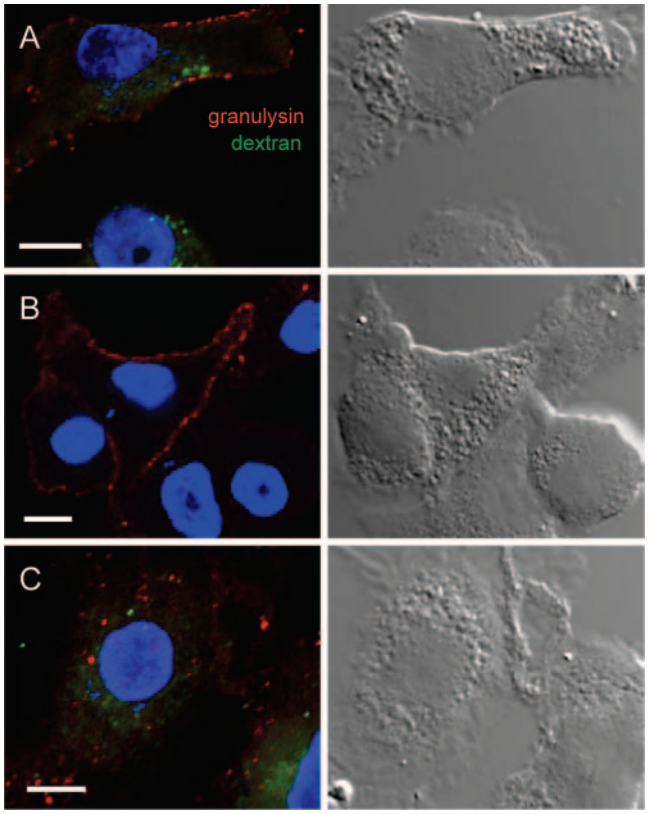

FIGURE 4. Cholesterol depletion prevents granulysin$_{132}$ uptake, but not binding. DC were pretreated with 20 mM MCD for 45 min before incubation with granulysin$_{132}$ (2.5 μM) and dextran-FITC (1 mg/ml) for 45 min at 37°C (*A*) or 4°C (*B*). *C*, Control cells were pretreated with serum-free medium for 45 min before incubation with granulysin$_{132}$ and dextran-FITC for 45 min at 37°C. Bars = 8 μm.

stained with a polyclonal Ab recognizing the EEA-1. Granulysin$_{132}$ was colocalized with the EEA-1 (Fig. 5*A*) in *L. innocua*-challenged DC after 10 min of incubation. After 60 min, the longest time analyzed for EEA-1 colocalization, granulysin$_{132}$ still resided in endosomes.

Immunostaining of *L. innocua*-challenged and granulysin$_{132}$-treated DC with an Ab recognizing LAMP-1 revealed that granulysin$_{132}$ clearly separated from the LAMP-1-positive compartment (Fig. 5*B*). DAPI-stained listerial DNA was found in the lysosomal, LAMP-1-positive compartment (arrowheads in Fig. 5*B*) or in phagosomes, which showed a distinct labeling for granulysin$_{132}$ (arrows in Fig. 5*B*). Positive staining of *L. innocua* for granulysin$_{132}$ remained detectable even when bacteria were isolated from granulysin$_{132}$-treated DC (data not shown). Overall, these results indicate that after uptake, granulysin is present in early sorting endosomes of DC and then transferred to phagosomes, where granulysin is able to bind *Listeria*.

Granulysin mediates lysis of L. innocua *in DC*

L. innocua-challenged DC were incubated with various concentrations of granulysin$_{132}$ or granulysin$_{145}$ without His-tag for 3 h at 37°C. The viability of the intracellular bacteria was tested in CFU assays (Fig. 6*A*). Granulysin$_{132}$ (5 μM) killed >41%, and 5 μM granulysin$_{145}$ killed >46% of the intracellular *L. innocua* after 3 h. Granulysin$_{132}$ (2.5 μM) was sufficient to lyse 29% of the intracellular *Listeria*, very similar to 2.5 μM granulysin$_{145}$, which killed 26%. Viability reduction of bacteria was strictly dependent on granulysin$_{132}$ dosage and did not occur with actin$_{frag}$ treatment.

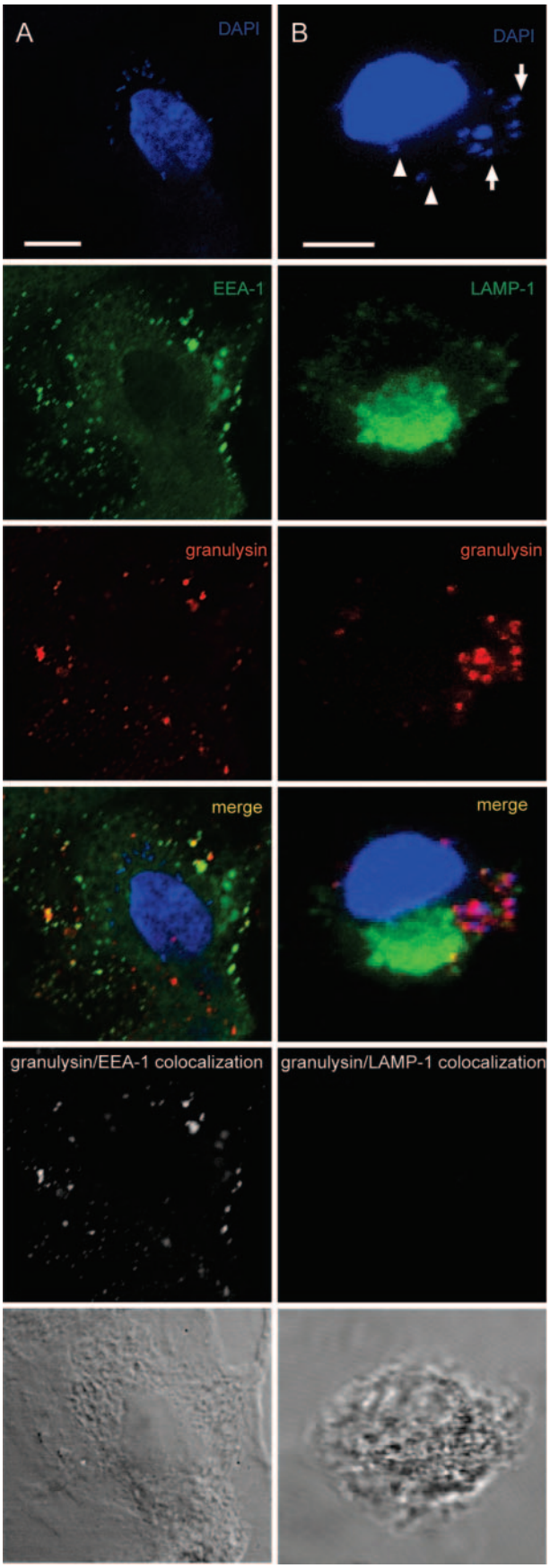

FIGURE 5. Granulysin$_{132}$ is localized in early endosomes and *Listeria*-containing phagosomes, but not in lysosomes. *A*, *L. innocua*-challenged DC were incubated with 2.5 μM granulysin$_{132}$ for 30 min at 37°C for colocalization with EEA-1. *B*, For double staining of granulysin$_{132}$ and LAMP-1, cells were treated for 90 min with 2.5 μM granulysin$_{132}$ at 37°C, then stained with a polyclonal anti-granulysin Ab and a polyclonal Ab recognizing EEA-1 or an anti-LAMP-1 mAb, respectively. Nuclear and bacterial DNA was labeled with DAPI (blue); early endosomes and lysosomes, respectively, are marked in green; and granulysin$_{132}$ is depicted in red. The yellow spots in the merge image (*A*) indicate colocalization of granulysin$_{132}$ with EEA-1. Colocalization of the green and red channels was additionally calculated using Imaris software. Arrowheads in *B* indicate listerial DNA spots that colocalize with LAMP-1, whereas arrows mark DNA spots that coincide with granulysin$_{132}$. Bar = 8 μm.

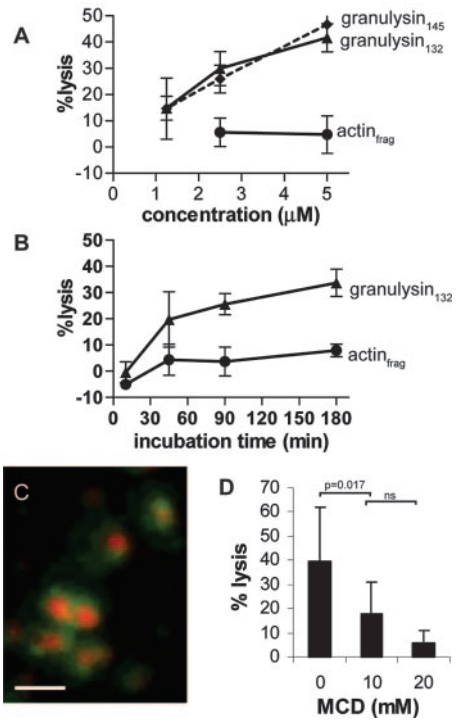

FIGURE 6. Granulysin$_{132}$ mediates lysis of *L. innocua* in DC. *L. innocua*-challenged DCs were incubated for 3 h with granulysin$_{132}$ in varying concentrations (*A*) or with 2.5 μM granulysin$_{132}$ for the indicated time periods at 37°C (*B*). After granulysin$_{132}$ incubation, cells were lysed in ice-cold water, and *Listeria* viability was assessed in CFU assays. The mean ± SD of four independent experiments are presented. *C*, Deconvoluted CLSM image of intracellular bacteria found in granulysin-treated DC. Bacterial DNA (red) is stained with DAPI, and granulysin$_{132}$ (green) is labeled with an anti-His Ab. Bar = 2 μm. *D*, *L. innocua*-challenged DC were cholesterol-depleted for 45 min with the indicated concentrations of MCD. Subsequently, DC were incubated with 2.5 μM granulysin$_{132}$ for 3 h at 37°C. After the incubation, cells were lysed, and bacterial viability was assessed in CFU assays. The mean ± SE of three independent experiments are presented. Statistical significance was calculated with Student's *t* test for paired samples.

Incubation of DC with granulysin$_{132}$ at 4°C for 3 h did not impair the viability of intracellular *L. innocua* (data not shown). To exclude bacteria loss due to detachment of perishing host cells, the viability of DC after granulysin$_{132}$ incubation was assessed. Granulysin$_{132}$ incubated in a concentration up to 5 μM had no effect on the viability of *L. innocua*-challenged DC monitored in lactate dehydrogenase release and thiazolyl blue (MTT) assays (data not shown).

Compared with the fast lysis of extracellular *L. innocua,* intracellular bacterial killing was delayed. Forty-five minutes of incubation at 37°C with 2.5 μM granulysin$_{132}$ was required to kill 12% of the intracellular bacteria (Fig. 6*B*). Intracellular lysis increased

to 25% after 90 min of treatment, but never reached the same level as in suspension (see Fig. 1*B*). The maximal reduction of 31% of the intracellular bacteria was achieved after 3 h of incubation with 2.5 μM granulysin$_{132}$. Deconvoluted high resolution CLSM images of *L. innocua* within granulysin$_{132}$-treated DC demonstrated bacterial DNA that was clearly surrounded by granulysin$_{132}$ labeling (Fig. 6*C*). Granulysin$_{132}$-mediated lysis of intracellular *L. innocua* was reduced after cholesterol depletion of DC by MCD in a dose-dependent manner (Fig. 6*D*). Preincubation of DC with 10 mM MCD diminished lysis of intracellular *L. innocua* to 13%, and only 4% of specific lysis was achieved after pretreatment with 20 mM MCD.

Discussion

In this study we could clearly demonstrate that recombinant granulysin was binding and dose-dependently killing *L. innocua* as free bacteria grown in suspension. Granulysin-induced lysis of *Listeria* is in agreement with data obtained by other groups using a variety of microbial pathogens, including *L. monocytogenes* (8, 20). To date, the binding of granulysin to bacteria has never been directly shown, but can be explained by properties of the SAPLIP family, of which granulysin is a member. SAPLIPs interact with a variety of lipids (14). Bacterial membranes contain mainly acidic phospholipids, such as phosphatidylglycerol and cardiolipin. Positive charges of granulysin (net charge, +11) can be assumed to be the driving force for binding to negatively charged bacterial membranes. Charges in granulysin are not homogeneously distributed, but are slightly polarized toward a region of the molecule corresponding to helix 3 (19). This region was proposed as the site of the initial contact of granulysin to bacterial membranes. After binding, granulysin is suggested to cluster at the bacterial membrane, leading to deformation and, subsequently, lysis by friction of adjacent granulysin molecules.

To reach intracellular bacteria, granulysin has to bind and enter mammalian cells that have apparent differences in the composition of membranes to prokaryotes. The outer leaflet of mammalian cells is mainly composed of zwitterionic phospholipids, such as phosphatidylcholine and sphingomyelin. Cholesterol is abundant in mammalian cells, but is absent in bacterial cell membranes. A third difference is the higher transmembrane potential of prokaryotic cells (40–43).

Binding of granulysin to mammalian cell membranes can again be explained by properties of the SAPLIP family. The interaction of saposins with sphingolipids has been extensively investigated. Dependent on the pH value, all saposins were reported to bind the negatively charged gangliosides, which are found enriched together with cholesterol in lipid rafts. In our study we could show by modifying arginine residues of granulysin that positive charges of arginine residues contribute crucially to the lytic activity against *L. innocua* as well as to the binding capacity to DC membranes. Lysine residues seem not to play an important role in lysis of bacteria and binding to eukaryotic cells. This is consistent with the finding that covering the granulysin arginine residues by butanedione reduced binding and lysis of *E. coli* (20). Granulysin was found bound to lipid rafts, as indicated by its colocalization with cholera toxin and CD55, both known markers for lipid rafts (25, 38, 44). Preincubation of cells with MCD or filipin, which are both known to deplete cholesterol (45), did not abolish binding of granulysin to lipid rafts. Mechanisms of granulysin binding to lipid rafts or even binding partners are still an enigma, but it is known to be cholesterol independent. Clustering of granulysin at ganglioside-rich regions of the plasma membrane could apply local shear stress that might trigger budding and subsequently endocytosis of granulysin-containing membrane vesicles. There is some evidence that local deformation and membrane tension contribute to the regulation of endocytosis (46, 47).

In contrast to binding, uptake of granulysin could be inhibited by cholesterol depletion, but also by incubation at 4°C. Inhibition of uptake at 4°C is a strong indication for an active process (48). Endocytosis of granulysin via the lipid raft/caveolae pathway is distinguished from both the clathrin-dependent and constitutive pinocytic pathways by its sensitivity to cholesterol depletion. In line with this observation is that dextran or transferrin as tracers for constitutive pinocytosis and clathrin-dependent pathway, respectively, were never found colocalized with simultaneously applied granulysin. After endocytosis, granulysin was found to be sorted differently from cholera toxin (49). Additional intracellular trafficking of granulysin from early endosomes may result in fusion of endosomes with phagosomes where granulysin was found to be colocalized with bacteria. Fusion of granulysin-containing early endosomes with phagosomes may be regulated by the small rab5 GTPase, which has been shown to be directly correlated with an accelerated maturation of *Listeria*-containing phagosomes (50).

In our model of *L. innocua*-challenged human DC binding, uptake and trafficking of granulysin to phagosomes as well as binding to and lysis of *L. innocua* were shown to occur in the absence of perforin. In contrast to these results, it was shown that *M. tuberculosis* was efficiently lysed in vitro in human DC by granulysin only in combination with perforin (8, 20). This combination of perforin and granulysin to achieve antibacterial activity against intracellular pathogens seems necessary at least occasionally. NKT cells and CTL could control mycobacterial infection by relying on granulysin, but not on perforin or Fas/Fas ligand interaction (11, 29). In vivo, it was found that perforin-negative, but granulysin-positive, $CD4^+$ cells are present at sites of bacterial infections (12, 13). This is in line with our in vitro data for granulysin activity. However, data are largely missing about the exact role of perforin in lysis of intracellular bacteria, e.g., *M. tuberculosis*. Data on perforin-dependent as well as independent uptake and activity of granulysin are not conclusive, and mechanisms of entry into host cells are poorly characterized. Perforin might well have a role in granulysin trafficking to compartments harboring intracellular bacteria. In contrast to apathogenic *L. innocua* pathogenic species such as *M. tuberculosis*, *Chlamydia spp.* and *Coxiella spp.* have evolved strategies to modify their intracellular compartments (51, 52) or are known to escape from vesicular compartments to the cytosol-like *L. monocytogenes* (53). Mycobactera, for example, can stop phagosome maturation by inhibiting acquisition of the rab5 effector EEA-1. By excluding this important regulator for vesicular trafficking, fusion processes within the endocytic pathway are impaired (54).

Overall, we propose a model of granulysin-mediated activity against intravacuolar bacteria. According to this model, binding due to electrostatic interactions and clustering of granulysin at membrane microdomains with elevated acidic sphingolipid content would lead to endocytosis as a first step toward lysis of intracellular bacteria. Such lipid raft microdomains were indeed found concentrated in the regions of the immunological synapse (55). After cholesterol-dependent uptake, granulysin is delivered to early sorting endosomes, fusing later with bacteria-harboring phagosomes, where the lysis of the bacteria is induced. Simultaneously secreted IFN-γ at sites of infection by the CTL would enhance granulysin targeting. There is evidence that activated macrophages up-regulate rab5 in response to IFN-γ (56). An elevated level of rab5 accelerates not only maturation of *Listeria*-containing phagosomes (50), but also the likelihood for phagosome-endosome fusion. Thus, the antimicrobial activity mediated by granulysin would be enhanced.

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Disclosures

The authors have no financial conflict of interest.

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Paper 2: Cholesterol in Negatively Charged Lipid Bilayers Modulates the Effect of the Antimicrobial Protein Granulysin

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Cholesterol in Negatively Charged Lipid Bilayers Modulates the Effect of the Antimicrobial Protein Granulysin

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Abstract. The release of granulysin, a 9-kDa cationic protein, from lysosomal granules of cytotoxic T lymphocytes and natural killer cells plays an important role in host defense against microbial pathogens. Granulysin is endocytosed by the infected target cell via lipid rafts and kills subsequently intracellular bacteria. The mechanism by which granulysin binds to eukaryotic and prokaryotic cells but lyses only the latter is not well understood. We have studied the effect of granulysin on large unilamellar vesicles (LUVs) and supported bilayers with prokaryotic and eukaryotic lipid mixtures or model membranes with various lipid compositions and charges. Binding of granulysin to bilayers with negative charges, as typically found in bacteria and lipid rafts of eukaryotic cells, was shown by immunoblotting. Fluorescence release assays using LUV revealed an increase in permeability of prokaryotic, negatively charged and lipid raft-like bilayers devoid of cholesterol. Changes in permeability of these bilayers could be correlated to defects of various sizes penetrating supported bilayers as shown by atomic force microscopy. Based on these results, we conclude that granulysin causes defects in negatively charged cholesterol-free membranes, a membrane composition typically found in bacteria. In contrast, granulysin is able to bind to lipid rafts in eukaryotic cell membranes, where it is taken up by the endocytotic pathway, leaving the cell intact.

Key words: Cholesterol — Lipid bilayer — Granulysin — AFM — Cytotoxicity — Lipid rafts

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Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells contribute to the host defense against intracellular pathogens by release of cytokines that activate antimicrobial effector pathways (Flynn et al., 1993) and lyse infected target cells by induction of apoptosis (Kaspar et al., 2001; Smyth et al., 2001) or by exocytosis of cytotoxic proteins, such as perforin and granulysin, which are stored in lysosomes (Kaufmann, 1999; Pena & Krensky, 1997). Granulysin is expressed constitutively in NK cells and cytotoxic T cells and upregulated following antigen-driven activation (Pena & Krensky, 1997; Stegelmann et al., 2005). It is released from the cytotoxic granules after cellular stimulation and is active against a broad range of intracellular pathogens, such as *Listeria monocytogenes, Mycobacterium tuberculosis* and *Trypanosoma cruzi* (Clayberger & Krensky, 2003; Krensky, 2000; Stenger et al., 1998; Walch et al., 2005).

Granulysin is a 9-kDa protein and belongs to the saposin-like protein (SAPLIP) family, which includes amebapores (Bruhn & Leippe, 1999), NK-lysin (Liepinsh et al., 1997) and saposins A, B, C and D (Clayberger & Krensky, 2003; Krensky, 2000; Morimoto et al., 1988, 1989; O'Brien & Kishimoto, 1991; Qi & Grabowski, 2001). The family members are cationic proteins, and they share a particular polypeptide motif of a five-helix bundle and highly conserved cysteine residues that form disulfide bonds (Munford, Sheppard & O'Hara, 1995), which give the molecule a stable structure. They interact with a variety of lipids, especially cholesterol (Vaccaro et al., 1995) and sphingolipids (Vaccaro et al., 1999). Comparing the amino acid sequences of the SAPLIP family members reveals that granulysin has the highest identity to NK-lysin (43% identity), a porcine protein with antibacterial activity (Andersson et al.,

1995). Although the SAPLIP family members have structural similarities, they have various biological functions: amebapores are capable of forming ion channels or pores in lipid membranes (Lynch, Rosenberg & Gitler, 1982; Young et al., 1982), saposin A-D modify the membrane to become substrates for enzymes and granulysin and NK-lysin appear to directly permeabilize bacterial membranes (Anderson et al., 2003; Krensky, 2000).

Recently, it has been found that granulysin binds to infected cells via lipid rafts (Walch et al., 2005), which are specialized membrane microdomains (Simons & Ikonen, 1997). Lipid rafts consist of phospholipids and cholesterol in the inner membrane leaflet and sphingomyelin and cholesterol in the outer membrane leaflet, and they are involved in a variety of cellular functions including endocytosis (Brown & London, 2000; Cambi et al., 2004; Manes, del Real & Martinez, 2003). Cholesterol has an important role in lipid rafts and in lipid bilayers, which usually exist in an ordered gel phase under the transition temperature of their lipids. However, cholesterol is able to eradicate the sharp transition between ordered gel phase and liquid crystalline phase, resulting in a liquid ordered phase, where the lipids have a high degree of lateral mobility as well as tightly packed acyl chains (Brown & London, 2000; McMullen & McElhaney, 1997; Pralle et al., 2000; Simons & Ikonen, 1997).

The lipid raft-bound granulysin is endocytosed and transferred via membrane vesicles to early endosomes and then to phagosomes. Subsequently, bacteria are killed by granulysin via membranolysis, whereas binding and intracellular trafficking of granulysin do not harm the target cell (Brown & London, 2000; Walch et al., 2005). Therefore, one important feature of granulysin is its ability to distinguish between prokaryotic and eukaryotic cells, which differ in the lipid composition of their membranes. A eukaryotic cell membrane consists of cholesterol, sphingomyelin and phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG), whereas the membrane of a prokaryotic cell lacks cholesterol and sphingomyelin, consisting mainly of cardiolipin, PC, PE, PS and PG, from which cardiolipin, PC, PE and PG are found in both membrane leaflets whereas PS is mainly located in the cytoplasmic leaflet (Gidalevitz et al., 2003; Kurz et al., 2005; Pomorski et al., 2004; Tannert et al., 2003).

Our hypothesis is that granulysin binds to and permeabilizes negatively charged phospholipid membranes typically found in bacteria but does not permeabilize target cell membranes when bound to lipid rafts or phospholipid membranes with eukaryotic lipid compositions. Experiments were performed to analyze the effect of granulysin on membranes with prokaryotic and eukaryotic lipid mixtures as well as on model membranes, which are commonly used to study the behavior of biological membranes (Bacia et al., 2004; Giocondi et al., 2004; Jass, Tjarnhage & Puu, 2000; Tong & McIntosh, 2004). We used membranes with negative and uncharged phospholipid compositions as well as lipid raft-like membranes. Fluorescence release assays were used to analyze the activity of granulysin and ultracentrifugation and immunoblotting, to study the binding of granulysin to membranes with different lipid compositions. To further investigate the effect of granulysin on membranes, atomic force microscopy (AFM) was used to visualize morphological changes of granulysin-treated membranes.

Our findings indicate a strong dependence on membrane lipid composition and cholesterol content for granulysin-mediated effects against bacterial and eukaryotic cell membranes.

Materials and Methods

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (POPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol, sphingomyelin from chicken egg yolk (SM) (Sigma-Aldrich, St. Louis, MO), 1,1′,2,2′-tetramyristoyl cardiolipin and *Escherichia coli* total lipid extract (Avanti Polar Lipids, Alabaster, AL) were stored in a chloroform:methanol (2:1) solution. The fluorescent lipid dye 2-(4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indancene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholinen-β-Bodipy (PC-β-Bodipy) was purchased from Molecular Probes (Eugene, OR), and water was obtained from a Millipore (Billerica, MA) water purification system.

Expression and Purification of Recombinant Granulysin

Recombinant granulysin and a fragment of human β-actin (actin fragment) were cloned, expressed and purified according to Walch et al. (2005). Briefly, histidine-tagged recombinant proteins were purified by nickel affinity chromatography (ÄKTA Prime; Amersham Biosciences, Uppsala, Sweden) using Ni-NTA agarose (Qiagen, Hilden, Germany). The eluted protein was diluted in 6 M guanidine-HCl and renatured in 0.75 M arginine, 0.05 M Tris-HCl (pH 8), 0.05 M KCl, 0.001 M ethylenediaminetetraacetic acid (EDTA) and 0.01 M oxidized dithiothreitol (Sigma-Aldrich) at a 1:10 dilution with constant stirring at 4°C for 48 h and further purified using Sep-Pac Vac 6cc (1 g) C_{18} cartridges (Waters, Milford, MA), from where it was eluted with 100% acetonitrile containing 0.1% trifluoroacetic acid and lyophilized. Protein concentration was determined using a Bio-Rad (Hercules, CA) protein assay, and purity was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Activity of recombinant proteins was tested according to Walch et al. (2005) using *Listeria innocua* in a turbidimetry assay.

Extraction of Membrane Lipids

Membrane lipids were extracted from *L. innocua*, Hep-2 cells and human erythrocytes according to the following procedure. Cells (20 ml erythrocytes from Buffy Coat; Blood Bank SRK, Zürich, Switzerland) and bacteria (1 liter Tryptic Soy Broth [TSB, Becton Dickinson, Le Pont de Claix, France] culture, optical density $[OD]_{600}$ = 0.8) were centrifuged at 500 × *g* for 10 min at 4°C, resolved in 10 ml sterile water, mixed with 20 ml isopropanol and incubated with occasional shaking for 1 h at room temperature. Chloroform (20 ml) was added to the solution, and the mixture was incubated for another hour at room temperature, followed by centrifugation at 500 × *g* for 30 min at 4°C. The lipid extract was mixed with 10 ml chloroform:methanol (2:1) and washed with 0.2 volumes of 0.05 M KCl. The purity of the lipids was tested by thin-layer chromatography, and the lipid extracts were stored at −20°C.

Preparing Large Unilamellar Vesicles and Supported Lipid Bilayers

E. coli, *L. innocua*, Hep-2 and erythrocyte lipid extracts, as well as negatively charged phospholipid (POPG:DPPC 1:2, POPG:DOPE 1:2, cardiolipin:DOPE 1:2 and POPG:SM:POPC 1:1:1), uncharged phospholipid (POPC:DPPC, 1:2) and lipid raft-like lipid mixtures (POPG:cholesterol:SM 1:1:1, POPG:cholesterol:POPC 1:1:1) were dissolved in 20 ml chloroform:methanol (2:1, final lipid concentration of approximately 0.5 mg/ml) and evaporated to dryness for 5 min at 500 mbar, 15 min at 250 mbar, 60 min at 150 mbar and 180 min at 100 mbar (Rotavapor; Büchi Labortechnik, Flawil, Switzerland). The dried lipid films were rehydrated in buffer (0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2) containing 12.5 μM of the fluorescent probe 8-aminonaphthalene-1,3,6-trisulfon acid, disodium salt (ANTS), and 45 μM of the collisional quencher *p*-xylene-bis-pyridinium bromide (DPX, Molecular Probes) by shaking with 220 rpm for 20 min at 47°C. For pH-dependence measurements, the dried lipid films were rehydrated in Pipes buffer at pH 5 and pH 7.2 (0.05 M Pipes, 0.15 M NaCl and 0.001 M $CaCl_2$). The liposome suspensions were extruded through a polycarbonate membrane with a pore diameter of 400 nm (Avestin, Ottawa, Canada) using a Liposofast extruder (Avestin) at 47°C for formation of large unilamellar vesicles (LUVs), which were purified from excess ANTS and DPX using gel filtration chromatography (10 desalting Grade [DG], Bio-Rad) and diluted to a final lipid concentration of 0.1–1 mg/ml.

Supported lipid bilayers were formed by depositing 80 μl LUV suspension onto a freshly cleaved mica surface with an area of 0.8 cm^2. After a 2 h incubation at 37°C for formation of patches of bilayers or at 50°C for testing the formation of bilayers with liquid crystalline and ordered gel phase separation, the specimens were cooled down to room temperature, gently rinsed with buffer and analyzed by AFM or confocal laser scanning microscopy.

Fluorescence Release Assays

Granulysin was incubated at various concentrations with LUV in 0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$ (pH 7.2) in a FluoroNunc 96-well plate (Nunc, Roskilde, Denmark). Cloned actin fragment and buffer were used as controls, and 0.1% Triton-X 100 (Sigma-Aldrich) was used for measurement of 100% lysis (fluorescence release, $FR_{100\%}$). ANTS fluorescence release was monitored in a DTX-880 multimode detector (Beckman Coulter, Fullerton, CA) at $\lambda_{excitation}$ = 370 nm, $\lambda_{emission}$ = 535 nm and the percentage fluorescence release was calculated using the formula $[(FR_{granulysin} - FR_{blank})/(FR_{100\%} - FR_{blank})]$ x 100.

Ultracentrifugation and Western Blot Analysis

LUVs were incubated in buffer solution (0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2) with 5 μM granulysin in a volume of 400 μl at 37°C for 2 h. For some experiments, granulysin was pretreated with 2,3-butanedione (Sigma-Aldrich), which covalently modifies the guanidine group of arginine residues (Walch et al., 2005). After incubation, the LUV solution was mixed with 1.2 ml 80% (w/v) sucrose solution, overlaid with 1 ml 50% sucrose (w/v) and 1 ml buffer solution. Ultracentrifugation was performed at 140,000 x *g* for 90 min at 4°C. Fractions (6 x 600 μl) were collected from the top and analyzed by Western blotting (tricine gel, 16.5%) using anti C-terminal-his antibody (1:5,000; Invitrogen, Carlsbad, CA) and a goat anti-mouse immunoglobulin G–peroxidase-conjugated antibody (1:5,000, Sigma-Aldrich). The signal was detected using an enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

AFM

Bilayers were imaged with a Bioscope Multimode AFM (Digital Instruments, Santa Barbara, CA) in contact mode in buffer solution containing 0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$ (pH 7.2) using NanoProbe Si_3N_4 cantilevers (Digital Instruments). The force was minimized by adjusting the set point to just under the jump-off point of the tip. The scan rate was typically 4–5 Hz. All images were obtained at room temperature (22°C). Images were analyzed using Nanoscope III (Digital Instruments) or Matlab (MathWorks, Natick, MA) software.

Supported lipid bilayers with different compositions were imaged with AFM, then treated for 2 h at 37°C with granulysin, cooled down to room temperature, rinsed gently with buffer (0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2) before imaging again. Real-time imaging could not be performed due to unspecific interactions of proteins in solution with the tip. Actin fragment and buffer were used as controls. For temperature stability experiments, granulysin-treated samples, after imaging with AFM, were heated to 47°C for 30 min, cooled down and imaged again.

The effect of antibody binding was visualized on previously imaged granulysin-treated bilayers by incubation with an anti C-terminal-His antibody (1:5,000) on a rocking platform for 1 h at room temperature, gently rinsed with buffer and subsequently imaged again.

Confocal Laser Microscopy

The changes in fluidity of PC-β-Bodipy containing negatively charged phospholipid bilayers were tested at room temperature and 47°C, which is over the transition temperature of the lipids, using fluorescence recovery after photobleaching (FRAP) trials with the confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany).

Results

Granulysin Permeabilizes Negatively Charged Cholesterol-Free Membranes

To analyze the permeabilization of vesicles by granulysin, ANTS-loaded LUVs of varying lipid compositions were treated with increasing concentrations of granulysin and the released ANTS fluorescence

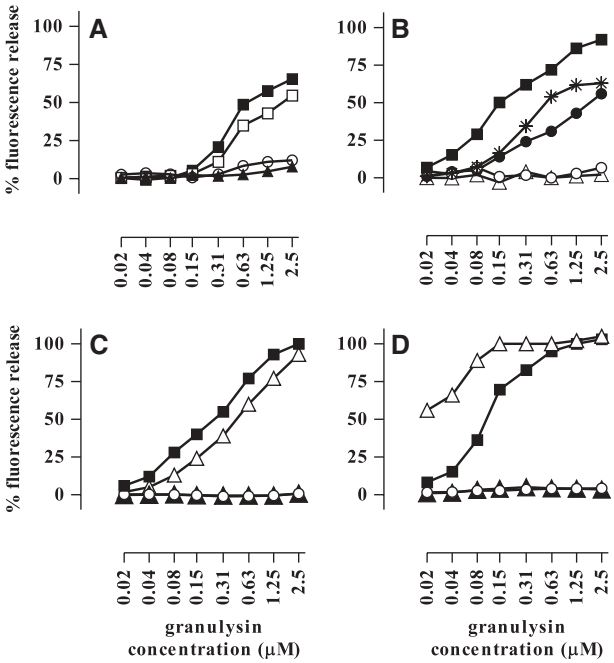

Fig. 1. Fluorescence release assays showing granulysin-induced effects on membranes with (*A*) *E. coli* (-■-), *L. innocua* (-□-), erythrocyte (-▲-) and Hep-2 (-○-) lipid mixtures and on (*B*) POPG:DPPC (-■-), cardiolipin:DOPE (-*-), POPG:DOPE (-●-) and uncharged POPC:DPPC (-○-) phospholipid LUVs. Negatively charged LUVs treated with actin fragment (-▲-) served as controls. (*C*) Granulysin-induced permeabilization on DPPC:POPG (-■-), lipid raft-like POPG:cholesterol:SM (-○-), POPG:SM:POPC (-Δ-) and POPG:cholesterol:POPC (-▲-). (*D*) Effect of pH on granulysin-induced permeabilization of lipid raft-like LUVs at pH 5 (-○-) and pH 7.2 (-▲-) and of negatively charged phospholipid LUVs at pH 5 (-Δ-) and pH 7.2 (-■-). One representative experiment out of three is shown.

was measured. First, we investigated vesicles composed of lipid extracts from prokaryotic and eukaryotic cell membranes. Only the vesicles composed of prokaryotic lipid mixtures were permeabilized by granulysin, whereas vesicles composed of eukaryotic lipid mixtures were not permeabilized (Fig. 1A). To compare the effect of granulysin on different lipid mixtures, PG and cardiolipin were used as negatively charged lipids and PC and PE as uncharged lipid throughout the following results. As shown in Figure 1B, LUVs containing negatively charged phospholipids were effectively permeabilized by granulysin in contrast to uncharged vesicles. A 50% permeabilization of POPG:DPPC-containing vesicles was reached at a concentration of 0.15 μM granulysin, cardiolipin:DOPE-containing vesicles at 0.63 μM of granulysin and POPG:DOPE-containing vesicles at 1.25 μM of granulysin. The actin fragment used as control could not permeabilize negatively charged LUVs.

To investigate the effect of granulysin on eukaryote-like vesicles, we tested the membranolysing effect of granulysin on cholesterol-containing lipid raft-like vesicles (POPG:cholesterol:SM). The results showed no permeabilizing of the cholesterol-containing vesicles. We also examined which of the membrane components prevented the membranolysing effect of granulysin, using vesicles containing phospholipids and cholesterol (POPG:cholesterol:POPC) or sphingomyelin (POPG:SM:POPC). Only cholesterol-free vesicles were permeabilized by granulysin. A 50% permeabilization of POPG:SM:POPC was reached at a granulysin concentration of 0.6 μM, whereas the cholesterol-containing vesicles were never permeabilized (Fig. 1C).

To further strengthen the assumption that charges are responsible for permeabilization of LUVs, the pH dependence of granulysin-induced fluorescence release was studied. Negatively charged phospholipid LUVs were treated with different concentrations of granulysin at pH 5 and 7.2. We found that granulysin-induced fluorescence release was more efficient at acidic than at neutral pH (Fig. 1D). A fluorescence release of 50% was reached with 0.02 μM granulysin at pH 5, much lower than the 0.15 μM measured at pH 7.2. This higher activity correlates with the increased positive charge of the molecule at lower pH. In contrast, granulysin was not able to permeabilize lipid raft-like LUVs (POPG:cholesterol:SM) even at pH 5.

Granulysin Binds to Negatively Charged and Lipid Raft-Like Membranes

Since granulysin was able to permeabilize vesicles composed of prokaryotic lipid compositions and negatively charged cholesterol-free vesicles, but not

membranes with eukaryotic lipid compositions and lipid raft-like vesicles (cholesterol-containing), binding of granulysin to vesicles with different compositions was studied. To this end, granulysin-treated LUVs were analyzed by ultracentrifugation and Western blotting. Interestingly, granulysin did not bind only to LUVs exhibiting distinct fluorescence release but to all LUVs containing negatively charged lipids, including lipid raft-like LUVs and LUVs composed of eukaryotic lipid mixtures (Fig. 2). Granulysin bound to LUVs was located in low-density fractions (fractions 1–2) and free granulysin in high-density fractions (fractions 4–6).

To study the importance of arginine residues for membrane binding, granulysin was pretreated with 2,3-butanedione (BAD) to block positively charged arginine residues. We found that BAD treatment abolished the binding of granulysin to negatively charged LUVs (Fig. 2H), supporting previous observations (Walch et al., 2005).

Granulysin Induced Defects on Supported lipid Bilayers

Due to the fact that granulysin binds to negatively charged phospholipid and all lipid raft-like LUVs but permeabilizes only negatively charged cholesterol-free LUVs, we visualized the granulysin-induced changes in supported lipid bilayers using AFM. LUVs were attached on mica, where they formed bilayers with irregularly shaped borders (Fig. 3) and diameters varying between 0.3 and 3 μm. The height of the bilayers was 4.5 ± 1 nm in accordance with the thickness of cell membranes (Chamberlain & Bowie, 2004). Domain separations could be visualized by AFM only in samples prepared over the transition temperatures for the lipids, at 50°C (*data not shown*). However, to obtain more contrast in the images by visualizing also the mica surface and not only the phospholipid bilayers, we prepared the samples at 37°C. This procedure offered the possibility to compare the heights of bilayers to those of the defects.

Untreated (Fig. 3A, C, E, H, J, L) and 5 μM granulysin-treated (Fig. 3B, D, F, I, K, M) lipid extract supported bilayers of various compositions were imaged by AFM. In prokaryotic and negatively charged phospholipid bilayers, distinct membrane defects were detected after treatment with granulysin (Fig. 3B, F, I). The depth of these defects was 5 ± 1 nm, indicating that they penetrated the entire bilayer. Untreated or actin fragment-treated negatively charged phospholipid bilayers never showed any defects (Fig. 3E, G). The bilayers composed of erythrocyte lipids and the cholesterol-containing lipid raft-like bilayers also showed no defects after treatment with granulysin (Fig. 3K, M).

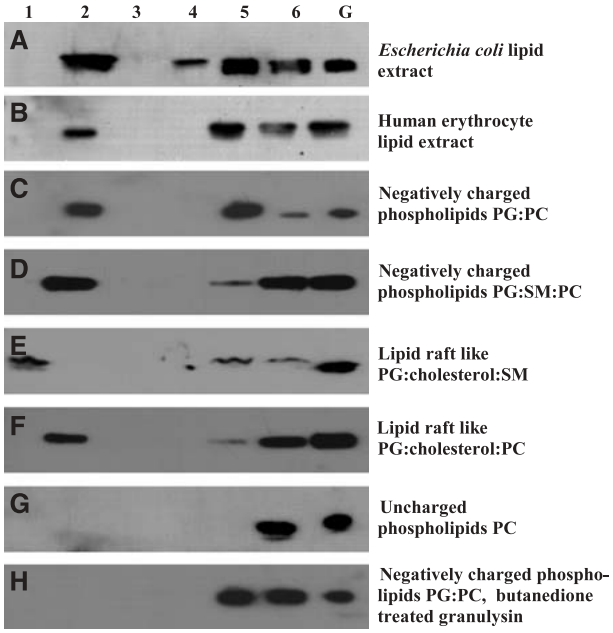

Fig. 2. Binding of granulysin (5 μM) to (*A*) LUVs composed of *E. coli* lipid extracts, (*B*) erythrocyte lipid extracts, (*C*) POPG:DPPC, (*D*) POPG:SM:POPC, (*E*) lipid raft-like POPG:cholesterol:SM, (*F*) POPG:cholesterol and (*G*) uncharged POPC:DPPC. (*H*) Binding of BAD-treated granulysin to negatively charged phospholipid LUVs. Binding was analyzed by ultracentrifugation and Western blotting. Fractions 1 and 2 contain LUVs with bound (*A-F*) or without (*G, H*) bound granulysin, and the unbound granulysin is in fractions 4, 5 and 6. The abbreviation G stands for recombinant granulysin without LUVs as a control.

Supported lipid bilayers incubated with granulysin were compared to granulysin-incubated LUVs after ultracentrifugation to examine if the defects were as well formed in bilayers of LUVs in suspension. After ultracentrifugation, granulysin-incubated LUVs formed supported bilayers on mica, showing the same irregularly shaped defects as before and only on cholesterol-free bilayers with negative charges (*data not shown*).

The concentration dependence of granulysin-induced defects was analyzed by AFM imaging of bilayers after incubation with 1 and 5 μM granulysin. The number of defects was counted on a representative area of 25 μm^2, and their diameters were measured using Nanoscope III software. The data were arbitrarily grouped in four size categories: ≤ 40, 41–80, 81–120 and ≥ 121 nm. Data are shown for defects on negatively charged phospholipid bilayers (POPG:DPPC and POPG:SM:POPC) in Figure 4. At a granulysin concentration of 5 μM, a higher percentage of defects were either 81–120 nm or larger than 121 nm when compared with defects on bilayers treated with 1 μM granulysin. These data indicate a distinct correlation between the concentration of granulysin and the size of the defects in supported bilayers.

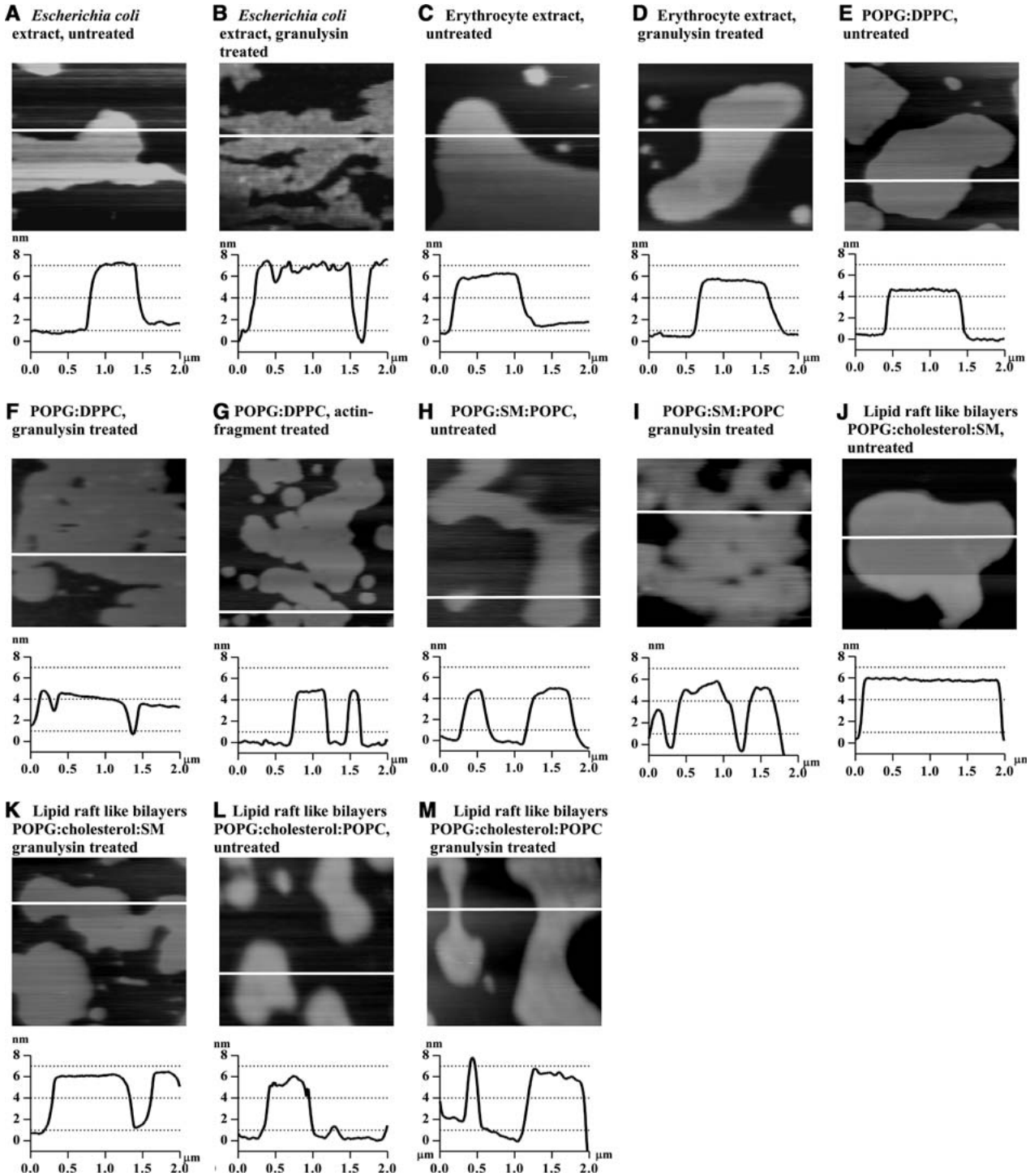

Fig. 3. AFM images of supported bilayers on mica, with a corresponding trace along the *white line* indicating the height images. Results show untreated (*A*) and 5 μM granulysin-treated bilayers composed of (*B*) *E. coli* lipid mixtures and untreated (*C*) and 5 μM granulysin-treated (*D*) erythrocyte lipid mixtures, (*E*) untreated, (*F*) 5 μM granulysin-treated and (*G*) 5 μM actin fragment-treated POPG:DPPC bilayers. (*H*) Untreated and (*I*) 5 μM granulysin-treated POPG:SM:POPC bilayers; (*J, K*) untreated and 5 μM granulysin-treated lipid raft-like POPG:cholesterol:SM bilayers, respectively; (*L, M*) untreated and 5 μM granulysin-treated POPG:cholesterol:POPC bilayers, respectively. Size of images: 2 x 2 μm, z-scale 15 nm.

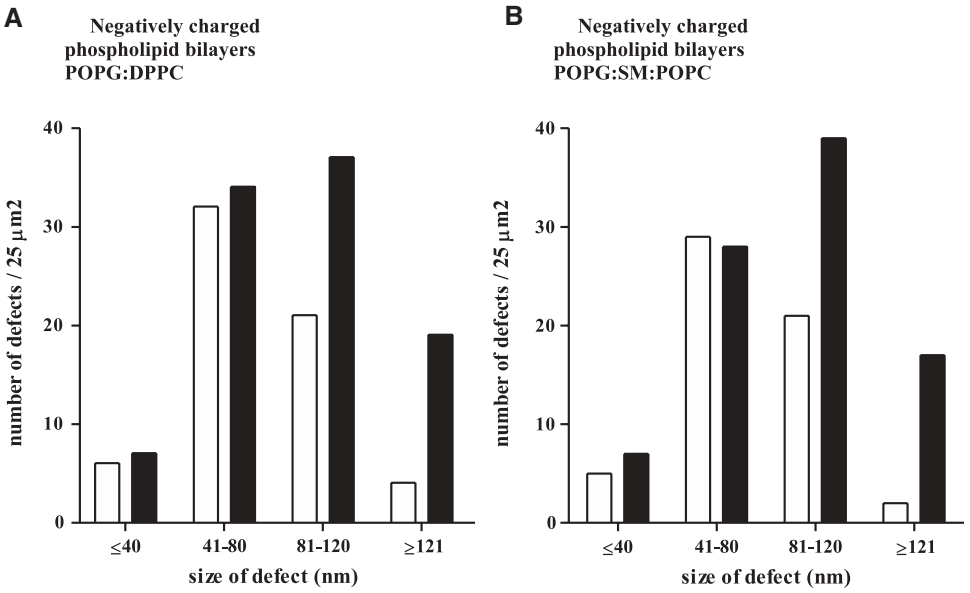

Fig. 4. Concentration dependence of granulysin-induced defects. The number of defects was calculated and the size measured on an area of 25 μm^2 of 1 μM (*open bars*) and 5 μM (*solid bars*) granulysin-treated bilayers from AFM images. The defects were classified into groups according to size: ≤40, 41–80, 81–120 and ≥121 nm. The results are shown for negatively charged phospholipid bilayers with (*A*) POPG:DPPC and (*B*) POPG:SM:POPC lipid mixtures.

Persistence of Granulysin-Mediated Defects

To test if granulysin-induced defects remain stable above the transition temperature of the lipids, FRAP experiments using confocal laser scanning microscopy at room temperature (Fig. 5A) and at 47°C (Fig. 5B) were performed. The result confirmed a higher recovery rate at higher membrane fluidity at 47°C. The persistence of granulysin-induced defects during higher membrane fluidity was tested on untreated negatively charged phospholipid bilayers (Fig. 5C) and on negatively charged phospholipid bilayers with granulysin-induced defects (Fig. 5D), which were heated to 47°C for 30 min and imaged again after cooling to room temperature by AFM (Fig. 5E, F). The results showed that the defects were still visible after the membrane was heated to over the transition temperature of the lipids (Fig. 5F).

Further evidence for the continuous and stable presence of granulysin was obtained by treatment of negatively charged phospholipid bilayers with granulysin and subsequently with anti-His-tag antibody. In granulysin-treated bilayers, the typical defects were visible (Fig. 6A). Following incubation with anti-His-tag antibody, the defects were no longer detectable. Instead, aggregates with a diameter of 20–120 nm and a height of 1.5 ± 0.5 nm were found (Fig. 6B). In untreated control specimens, the surface of the bilayers remained smooth also after antibody incubation (Fig. 6C).

Discussion

In spite of the growing interest in antimicrobial proteins and their interactions with membranes, the structure-related function and mechanism involved in the contact between granulysin and prokaryotic *vs.* eukaryotic cell membranes remains unclear. Recently, we showed that granulysin destroys intracellular bacteria, such as *L. innocua*, after binding to and uptake via lipid rafts by the target cell (Walch et al., 2005). This mechanism ensures that no premature killing of the infected cell occurs with resulting release and further spreading of intracellular bacteria.

To this end, granulysin must interact differently with eukaryotic *vs.* prokaryotic cell membranes. These membranes differ in their physical and chemical properties. The major membrane components of prokaryotic cells are phospholipids, and their membranes contain higher amounts of anionic lipids than eukaryotic cell membranes but no cholesterol or SM (Gidalevitz et al., 2003; Huijbregts, de Kroon & de Kruijff, 2000; Simons & Ikonen, 1997; Simons & Vaz, 2004; Slotte, 1999; Tannert et al., 2003). This seems to be of fundamental importance for the membrane interaction, activity and selectivity of antimicrobial peptides, as we could show for granulysin in the present study. The behavior of granulysin, when coming in contact with eukaryote- and prokaryote-like membranes, was mirrored by the use of cell or bacterial lipid extracts. The model membranes consisted of phospholipids and sterols. They were chosen for their biological interest of being a component of the eukaryotic or prokaryotic membrane or for their ability to stabilize the model system and simplify imaging. Our experiments on LUVs revealed that granulysin binds to cholesterol-containing membranes, generally found in eukaryotic membranes, but is not able to permeabilize them, confirming the observation made on infected cells (Walch et al., 2005), whereas granulysin is able to bind and permeabilize prokaryotic membranes, as we showed for *E. coli* and *L. innocua* membranes, and

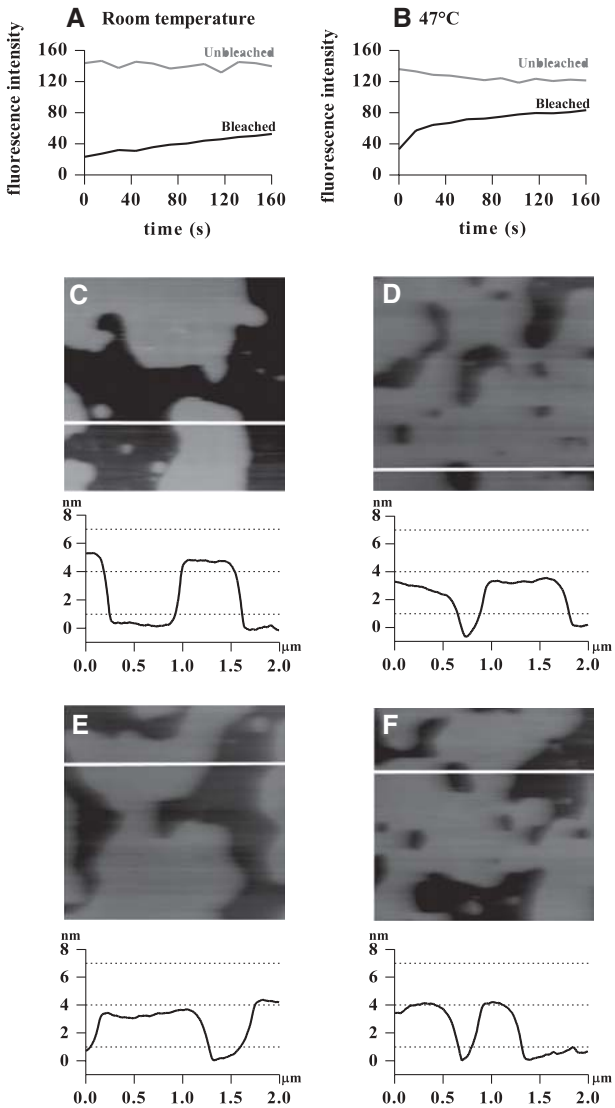

Fig. 5. The level of fluidity of phospholipid membranes was tested by incorporating a fluorescent dye (PC–β–Bodipy) into the bilayer and performing FRAP experiments using confocal laser scanning microscopy. (*A, B*) Fluorescence recovery compared to a control area at room temperature and at 47°C, respectively. Persistence of the granulysin-induced defects was tested by imaging (*C*) untreated and (*D*) 5 µM granulysin-treated negatively charged phospholipid bilayers and then heating the (*E*) untreated and (*F*) treated bilayers over the transition temperature (47°C) before imaging again in AFM. Size of images: 2 x 2 µm, z-scale 15 nm.

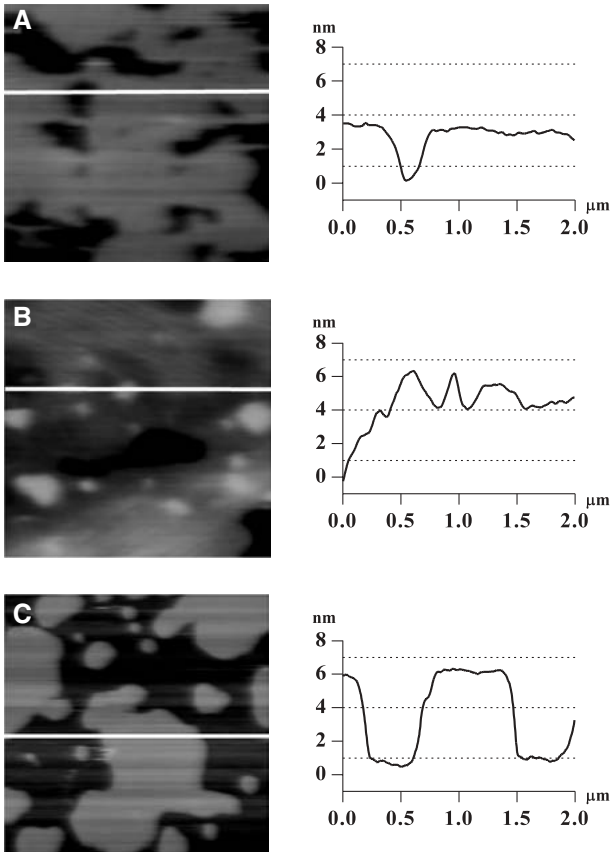

Fig. 6. Detection of granulysin binding with anti-His-tag antibody. Supported negatively charged phospholipid bilayers were incubated with 5 µM granulysin and imaged via AFM showing typical defects (*A*). The specimens which were subsequently incubated with anti-His-tag antibody displayed various aggregates of 1.5 ± 0.5 nm height (*B*). The surface of untreated bilayers remained smooth after antibody incubation (*C*). Size of images: 2 x 2 µm, z-scale 15 nm.

cholesterol-free negatively charged phospholipid model membranes. Our results regarding the activity of granulysin against prokaryotic membranes are in agreement with a recent study by Ramamoorthy et al. (2006). They showed that a granulysin-derived peptide, G15, is able to bind and disrupt *E. coli* outer membranes. They also demonstrated that G15 is not able to bind to cholesterol-containing (POPC:POPG:cholesterol) membranes. We could show that recombinant granulysin binds to all negatively charged membranes, as well as cholesterol-containing lipid raft-like membranes, typically found in eukaryotes. This indicates that other domains must be responsible for binding to cholesterol-containing negatively charged membranes.

The essential components in the formation of lipid rafts are cholesterol and SM (Simons & Ikonen, 1997), which form a hydrogen bond between the 3β-OH group of cholesterol and the amide group of SM. This hydrogen bond is responsible for the tight packing of lipid rafts but still ensures a high degree of lipid mobility (Brown, 1998; Mombelli et al., 2003). The tight packing in liquid ordered phase of lipid rafts can additionally be formed between cholesterol and phospholipids (Ohtake, Schebor & de Pablo, 2006). The acyl chains between the phospholipids are held together by van der Waals interactions, which are considerably weaker than hydrogen bonds and, therefore, responsible for a less tight membrane packing of prokaryotic membranes and non-lipid raft parts on the eukaryotic cell membrane (Niu &

Litman, 2002), which contain less cholesterol than lipid rafts (Arispe & Doh, 2002).

We also found that granulysin is more active in permeabilizing negatively charged phospholipid LUVs at lower pH values and that BAD-treated granulysin, due to the modified arginine residues, cannot bind to negatively charged vesicles, in agreement with the different positive charge of the protein at various pH values. At neutral pH granulysin has a net charge of 8, whereas at pH 5 the net charge is 10. Hanson et al. (1999) showed previously, using fluorescence release assays, that granulysin acts in a pH-dependent manner; but, in contrast to our results, they found that granulysin is less active at lower pH values. Our data indicate that the protection from lysis of granula may stem from the lipid composition of the membrane; therefore, we suggest this to be part of a mechanism for inhibiting the permeabilization of granules by granulysin. In addition, the pH-dependent activity might have a function in modulating the activity of granulysin in early and late endosomes, as well as in lysosomes and phagolysosomes, all defined stages of endocytosis. The pH in these compartments changes from 5.9–6.0 in early endosomes to 5.0–6.0 in late endosomes to 5.0 in lysosomes (Geisow & Evans, 1984; Renswoude et al., 1982). These different pH values could convert granulysin from a less active to a highly active protein at low pH in the later stages of endocytosis, further promoting binding to and killing of intracellular pathogens. It is interesting to note that trafficking of intracellular bacteria in the host cell, e.g., *L. innocua*, is along the same route, namely from endosomes via phagosomes to phagolysosomes, where it can be efficiently lysed by granulysin (Gaillard et al., 1987). In the same endocytic pathway, in the phagosomes, *M. tuberculosis* can be killed (Armstrong & Hart, 1971).

The permeabilization of bacterial membranes after binding of granulysin is supposed to originate from oligomerization of the protein. Similar to other cationic proteins of the SAPLIP family, the three-dimensional structure of granulysin does not allow the prediction of a pore (Anderson et al., 2003). Anderson et al. (2003) propose a model for the mechanism of action for granulysin. After oligomerization, friction between granulysin molecules results in cooperative membrane lysis and each granulysin molecule binds to its neighboring molecules, applying local forces to a part of the membrane. This model correlates with the carpet model, where, first, protein molecules cover the infected cell by binding to the membrane using electrostatic interactions and, second, permeabilization of the membrane is induced only where the protein concentration is high enough (Anderson et al., 2003; Pouny et al., 1992; Shai, 1999).

These model predictions are in agreement with our results. Granulysin induces defects on cholesterol-free membranes in a concentration-dependent manner, which is in accordance with the oligomerization of the protein bound to the bacterial membrane. This is further supported by the observation of the nonhomogeneous binding of His-tag antibodies to granulysin-treated membranes. Binding of the antibody also proves that granulysin stays attached to the membrane after causing defects. Furthermore, higher membrane fluidity, as shown by varying the temperature, does not lead to granulysin dissociation from the membranes. The observed persistence of granulysin-induced defects during transient fluidity changes over the transition temperatures of the lipids suggests stable protein-protein and/or protein-lipid interactions causing the defects to stay permanently.

In conclusion, our study showed that the composition and the physical properties of the membrane are important for granulysin to interact with infected eukaryotic host cells without affecting the viability of the cell. The cholesterol content of the host cell membrane plays a particularly important role in the potential of granulysin to perturb the membrane. Not only lipid rafts but also nonraft parts of the eukaryotic membrane contain cholesterol and can therefore not be permeabilized, whereas granulysin is able to bind to lipid rafts from where the protein is taken up via endocytosis. The cholesterol-free composition of the prokaryotic membrane is crucial for the interaction of granulysin with intracellular pathogens, leading to permeabilization and finally lysis of the bacteria. This provides important information for understanding the function and mechanism of action of granulysin and other positively charged antimicrobial proteins and how they interact with eukaryotic and prokaryotic cell membranes.

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Paper 3: Reversible Binding and Oligomerization of the Antimicrobial Protein Granulysin on Prokaryotic and Eukaryotic Membranes (In progress)

Reversible Binding and Oligomerization of the Antimicrobial Protein Granulysin on Prokaryotic and Eukaryotic Membranes

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ABSTRACT The release of the cytotoxic protein granulysin from lysosomal granules of cytotoxic T lymphocytes and natural killer cells plays a central role in host defence against microbial pathogens. A key molecule killing intracellular bacteria is granulysin which binds to the infected target cells, and is endocytosed via lipid rafts. After transfer via endosomes to bacteria containing compartments granulysin is transferred to the bacterial cell membrane. By largely unknown mechanisms granulysin is inducing lysis of bacteria by a possible oligomerization. On prokaryotic and eukaryotic membrane a fraction of granulysin was found to transiently bind by surface plasmon resonance experiments. Fluorescent resonance energy transfer analysis of fluorescently labeled granulysin revealed oligomerization on all tested membranes. The fraction of irreversible bound and oligomerized granulysin was smaller on eukaryotic compared to prokaryotic like membranes. Based on these results we conclude that granulysin binds transiently to and a part of the bound granulysin oligomerizes on negatively charged membranes. Transient binding leads to uptake of soluble granulysin that subsequently oligomerizes and permeabilizes bacterial membranes.

Introduction

The antimicrobial protein granulysin is produced by human cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, which contribute to the host defence against intracellular pathogens by exocytosis of lysosomal granules containing granulysin and other cytotoxic

proteins, such as perforin and granzyme B (Kaufmann, 1999; Pena and Krensky, 1997). Granulysin is expressed constitutively in NK cells and CTLs, and is up-regulated following antigen driven activation (Pena and Krensky, 1997; Stegelmann et al., 2005). It is released from the cytotoxic granules after cellular stimulation and is active against a broad range of intracellular pathogens, such as *Listeria monocytogenes, Mycobacterium tuberculosis* and *Trypanosoma cruzi* (Clayberger and Krensky, 2003; Krensky, 2000; Stenger et al., 1998; Walch et al., 2005).

Granulysin belongs to the saposin-like protein (SAPLIP) family, which includes amoebapores (Bruhn and Leippe, 1999), NK-lysin (Liepinsh et al., 1997) and saposin A, B, C and D (Clayberger and Krensky, 2003; Krensky, 2000; O'Brien and Kishimoto, 1991; Qi and Grabowski, 2001; Sasaki et al., 1998). These cationic proteins share a particular polypeptide motive of a five-helical bundle and highly conserved cysteine residues that form disulphide bonds (Munford et al., 1995). They interact with a range of lipids, particularly cholesterol (Vaccaro et al., 1995) and sphingolipids (Vaccaro et al., 1999).

We showed in a previous study that granulysin interacts differently with eukaryotic and prokaryotic membranes (Barman et al., 2006), due to the differences in the membrane lipid compositions. An eukaryotic cell membrane consists of cholesterol, sphingomyelin and phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylglycerol (PG), whereas the membrane of a prokaryotic cell lacks cholesterol and sphingomyelin, consisting mainly of cardiolipin, PC, PE, PS and PG (Gidalevitz et al., 2003; Pomorski et al., 2004; Simons et al., 1992; Tannert et al., 2003). To understand differences in the permeabilization capacity (Barman et al., 2006) and the mechanism of uptake and subsequent permeabilization of bacteria by granulysin and other antimicrobial proteins, it is crucial to test the binding activity of granulysin on eukaryotic and prokaryotic membranes.

After the initial binding to the membrane of the infected host cell, granulysin is taken up via lipid raft mediated endocytosis and subsequently has to reach the intracellular bacteria, bind to their negatively charged, cholesterol free membrane and finally lyse the bacteria (Barman et al., 2006; Walch et al., 2005). We visualized using the atomic force microscope the defects caused by granulysin on cholesterol free membranes mimicking bacterial membranes (Barman et al., 2006). The size of these defects is mainly between 40 nm and 120 nm, too large for being caused by a single molecule, suggesting that these defects could be a result from an oligomerization of the protein.

Other cytotoxic proteins, such as streptolysin O, also forms oligomers to be active (Walev et al., 1995). It has been shown that this toxin first binds in a monomer form to lipid bilayers, then the molecules collides with one another, leading to a formation of non-covalently bonded polymeric aggregates (Alving et al., 1979; Johnson et al., 1980; Walev et al., 1995). Also saposin c, which belongs to the SAPLIP family, has been shown to be able to bind to the membrane and then several saposin c molecules are needed to penetrate the membrane, which leads to membrane reorganization (You et al., 2003; You et al., 2004).

Our hypothesis is that granulysin binds to the eukaryotic, partly oligomerizes on the membrane when still a large fraction of the protein stays in a monomeric form, enters then the cell by endocytosis, subsequently binds to and oligomerizes on the bacterial membrane, leading to permeabilization and lysis of the bacteria. We used membranes with negative and uncharged phospholipid compositions, but also membrane extracts from eukaryotic and prokaryotic cells. Surface plasmon resonance experiments showed the binding activity of granulysin, and immunoblotting revealed the oligomerization of granulysin on membranes with different lipid compositions. To further confirm the oligomerization of granulysin on membranes, fluorescence resonance energy transfer experiments were performed.

Our findings indicate a strong dependence on the membrane lipid compositions for granulysin to be able to bind to membranes but also that the binding of the protein is transient. Additionally we showed that after the initial binding, granulysin oligomerizes on the membrane of both prokaryotic and eukaryotic cells.

Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-glycerol) (POPG), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC), cholesterol (Sigma-Aldrich, St. Louis, MO) and *Escherichia coli* total lipid extract (Avanti Polar Lipids, Inc., Alabaster, AL) were stored in a chloroform:methanol (2:1) solution. Streptolysin O (Sigma-Aldrich) was stored in 0.15 M Tris, pH 7.2 in -80°C, and activated before use with 0.025 M L-Cysteine (Sigma-Aldrich) for 10 minutes at 37°C. Water was obtained from a Millipore water purification system (Millipore, Billerica, MA).

Extraction of Membrane Lipids

Membrane lipids were extracted from human erythrocytes according to the following procedure. Cells (20 ml erythrocytes from Buffy Coat, Blood Bank SRK Zürich, Switzerland), were centrifuged at 500 x g for 10 minutes at 4°C, resolved in 10 ml sterile water, mixed with 20 ml isopropanol and incubated with occasional shaking for 1 hour at room temperature. Chloroform, 20 ml, was added to the solution and the mixture was incubated another hour at room temperature, followed by a centrifugation at 500 x g for 30 minutes at 4°C. The lipid extract was mixed with 10 ml chloroform:methanol (2:1) and washed with 0.2 volumes of 0.05 M KCl. The purity of the lipids was tested by TLC and the lipid extracts were stored at -20°C.

Preparing large unilamellar vesicles (LUV)

Escherichia coli and erythrocyte lipid extracts, as well as negatively charged phospholipids with cholesterol (POPG:DPPC:Cholesterol 1:1:1) and without cholesterol (POPG:DPPC 1:2) (Shany et al., 1974) as well as uncharged phospholipids with cholesterol (POPC:DPPC:Cholesterol 1:1:1) and without cholesterol (POPC:DPPC 1:2) were dissolved in 20 ml chloroform:methanol (2:1) (final lipid concentration of approximately 0.5 mg/ml) and evaporated to dryness for 5 minutes at 500 mbar, 15 minutes at 250 mbar, 60 minutes at 150 mbar and 180 minutes at 100 mbar (Rotavapor, Büchi Labortechnik, Flawil, Switzerland). The dried lipid films were rehydrated in buffer (0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2) with or without 12.5 μM of the fluorescent probe 8-aminonaphtalene-1,3,6-trisulfon acid, disodium salt (ANTS) and 45 μM of the collisional quencher p-xylene-bis-pyridinium bromide (DPX) (Molecular Probes) by shaking with 220 rpm for 20 minutes at 50°C. The liposome suspensions were extruded through a polycarbonate membrane with a pore diameter of 400 nm (Avestin, Ottawa, ON, Canada) using a Liposofast extruder (Avestin) at 50°C for formation of large unilamellar vesicles, which were purified from excess ANTS and DPX using gel filtration chromatography (10 Desalting Grade, Bio-Rad Laboratories) and diluted to a final lipid concentration of 0.1 - 1 mg/ml.

Expression, Purification and Labelling of Recombinant Proteins

Recombinant granulysin and a fragment of human β-actin (actinfragment) were cloned, expressed and purified according to Walch et al. (2005). Briefly, histidine-tagged recombinant proteins were purified by nickel affinity chromatography (ÄKTA prime, Amersham Biosciences, Uppsala, Sweden) using Ni-NTA agarose (Qiagen, Hilden, Germany). The

eluted protein was diluted in 6 M guanidine-HCl and renatured in 0.75 M arginine, 0.05 M Tris-HCl (pH 8), 0.05 M KCl, 0.001 M EDTA and 0.01 M oxidized dithiothreitol (Sigma-Aldrich) at a 1:10 dilution with constant stirring at 4°C for 48 hours and further purified using Sep-Pac Vac 6cc (1 g) C_{18} cartridges (Waters, Milford, MA), from where it was eluted with 100% acetonitrile containing 0.1% trifluoroacetic acid and lyophilized. Protein concentration was determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and the purity was estimated by SDS-PAGE. Activity of recombinant proteins was tested according to Walch et al. (2005) using *Listeria innocua* in a turbidimetry assay. Recombinant Saposin C was purified according to Qi and Grabowski (2001).

Labeling Recombinant Granulysin with Alexa Fluor 488 and Alexa Fluor 595

To label recombinant granulysin with Alexa Fluor 488 carboxylic acid or Alexa Fluor 594 carboxylic acid (succinimidyl esters, Molecular Probes, Eugene, OR), a 2 liter *E. coli* culture was resuspended in 40 ml 0.3 M NaCl and 0.05 M NaH_2PO_4 with pH 8, lysed by adding 1 mg/ml lysozyme, 1% Triton X-100, 50 µg/ml DNase, and 5 µg/ml RNase (Sigma-Aldrich), and bound to 250 µl Ni-NTA (Qiagen) over night in +4°C. Unbound proteins were washed away, the buffer was exchanged to 0.1 M $NaHCO_3$ pH 8.3 and granulysin bound to the agarose was incubated over night at +4°C with 50 µl Alexa Fluor dye (2 mg/ml). The labelled protein was eluted with 0.3 M NaCl, 0.05 M NaH_2PO_4 and 0.2 M Imidazole, pH 8 and purified using reverse phase chromatography HPLC (Agilent Technologies Deutschland GmbH, Waldbronn, Germany), fractions were analysed by mass spectroscopy at the Functional Genomics Center in Zurich and only single labeled granulysin molecules were used for Fluorescence Resonance Energy Transfer experiments.

Fluorescence Resonance Energy Transfer (FRET)

The FRET signal of Alexa Fluor labeled granulysin was measured with a Fluorolog 4 (Horiba Jobin Yvon Inc, Edison NJ), by exciting at 490 nm and measuring the emission spectra from 500 nm to 800 nm. The experiments were performed in a quartz cuvette with a volume of 130 µl. First 130 µl of a liposome solution was measured as a blank, then 1 µM Alexa Fluor 488 labeled granulysin was added and finally 1 µM Alexa Fluor 594 labeled granulysin. As control both labeled granulysins were mixed together and measured in buffer solution (0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2).

For the FRET experiments performed using the confocal laser scanning microscope (SP5, Leica Microsystems, Mannheim, Germany), the bilayers were formed in a flowcell pretreated

with poly-L-lysin. Granulysin labeled with Alexa Fluor 488 was bound to the membrane using a flow of 30 µl/min and the signal was recorded at excitation wavelength 490 nm and emission 520 nm. Then granulysin labeled with Alexa Fluor 594 was bound to the same membrane using the same flowrate, and the flow was controlled using free Cy5 dye in the solution, and the FRET signal was measured at emission wavelength 620 nm.

Surface Plasmon Resonance

Surface plasmon resonance experiments were performed with a Biacore 3000 (Biacore AB, Uppsala, Sweden) at the Functional Genomics Center at the University of Zürich using an L1 chip (Biacore AB) in a buffer containing 0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2. The flowcells were coated with 150 µl vesicles with a flow rate of 5 µl/min. The experiment was perfomed by comparing the binding of different concentrations of granulysin and the control proteins on these bilayers for an association time of 90 seconds and dissociation of 240 seconds at a flow rate of 60 µl/min.

SDS-PAGE and Western Blotting

Liposomes were incubated 1 hour together with 1 µM granulysin, mixed with either reducing or nonreducing sample buffer (0.2 M Tris, 40% glycerol, 4% sodium dodecyl sulphate, 0.04% bromophenol blue, with or without 4% β-mercaptoethanol). Reduced and nonreduced samples were analyzed by western blotting (15% acrylamide or 4-20% gradient acrylamide) using anti C-terminal-his antibody (1:5000, Invitrogen, Carlsbad, California) and a goat-anti-mouse IgG–peroxidase-conjugated antibody (1:5000, Sigma-Aldrich). The signal was detected using ECL plus western blotting detection system (Amersham Biosciences).

Results

Interaction of Granulysin with Immobilized Vesicles

To analyze the binding of granulysin to bilayers, negatively charged and uncharged cholesterol containing LUV were immobilized on a Biacore L1 chip (Biacore AB). Granulysin in various concentrations (10 µM, 1 µM and 0.1 µM) was added to the bilayers formed on a L1 chip and the difference in the binding between the negatively charged test surface (POPG:DPPC:Cholesterol 1:1:1) and the uncharged control surface (POPC:DPPC:Cholesterol 1:1:1) was compared. The results are plotted in Figure 1A, which showed that granulysin bound in a concentration dependent manner to negatively charged cholesterol containing bilayers, in comparison to uncharged bilayers. Also the rate of

association of granulysin was different depending on the concentration and the shape of the association curve was dependent on the amount of granulysin. The higher the concentration of the protein, the faster it bound to the negatively charged membrane until equilibrium was reached. During the dissociation period when granulysin was removed from the buffer part of the bound material detached meaning that a part of granulysin was reversibly bound and possibly some lipids were removed from the bilayers. An irreversibly bound fraction of granulysin did not come off during the dissociation period, and this fraction of the protein was also dependent on the initial concentration of granulysin. As a control protein a fragment of human β-actin was used. This protein did not show a binding to these bilayers (Figure 1A). To see if sequential loading of granulysin to the negatively charged surface (POPG:DPPC:Cholesterol 1:1:1) leads to higher amounts of bound proteins or a changed binding behavior three binding and dissociation cycles were performed.. Granulysin was bound and detached to the bilayers during each cycle with identical binding kinetics (Figure 1B) but with a lower net increase of granulysin irreversibly bound to the bilayer.

Interaction of Granulysin with Prokaryotic and Eukaryotic Membranes

To further study the binding abilities of granulysin on prokaryotic and eukaryotic membranes, vesicles made of lipid extracts of *E. coli* and human erythrocytes were used. These vesicles were immobilized on a L1 chip and the binding of 10 μM granulysin on these membranes was analyzed using the Biacore 3000, and compared with the binding of granulysin to uncharged phospholipid vesicles (POPC:PDDC:Cholesterol 1:1:1). The result showed clearly that granulysin is able to bind to both eukaryotic and prokaryotic membranes (Figure 2), and that it binds more to the prokaryotic membrane. Part of the protein was only reversibly bound and detached during the dissociation period. The rate of dissociation of the protein was slower compared to the dissociation of the protein bound to artificial phospholipid bilayers, but a part still bound irreversibly to the membrane (Figure 1).

Interaction of Saposin C and Streptolysin O with Immobilized Vesicles

To compare the binding of granulysin two other membrane active proteins, saposin C and streptolysin O, were analyzed using the Biacore 3000. Negatively charged (POPG:DPPC:Cholesterol and POPG:DPPC:POPC) as well as uncharged control vesicles (POPC:DPPC:Cholesterol) for saposin C and cholesterol free control bilayer (POPG:DPPC 1:2) for streptolysin O were immobilized on an L1 chip and the association of saposin C and streptolysin O with these bilayers was studied using the Biacore 3000. Saposin C showed a negative response on the sensogram, when comparing the association of 10 μM of the protein

with the two different bilayers (Figure 3A). This means that lipids are coming off from the negatively charged membrane. Streptolysin O (2500 U/ml) showed a similar binding ability to the cholesterol containing bilayer as 1µM granulysin with fast initial binding, but the dissociation rate was noticeably slower than for granulysin (Figure 3B), revealing a more tight binding to the membrane compared to granulysin. As a control membrane for the binding study of streptolysin O, cholesterol free negatively charged membranes were used. Streptolysin O is known to bind to the membrane cholesterol (Shany et al., 1974).

Oligomerization of Granulysin on Phospholipid Membranes

We showed earlier using an atomic force microscope that granulysin forms defects with a size ranging from 40 nm to 120 nm on negatively charged cholesterol free supported lipid bilayers simulating the bacterial membrane (Barman et al., 2006). To prove the hypothesis that granulysin oligomerizes which then would lead to defects on membranes and permeabilization; we used fluorescence resonance energy transfer (FRET) experiments. To this end granulysin was labeled with either an Alexa Fluor 488 or an Alexa Fluor 594 and were analyzed during interaction withwith phospholipid vesicles (POPG:DPPC 1:1) by exciting at 490 nm and measuring the emission spectrum from 500 nm to 800 nm. The volume of the experiment was kept low (130µl), due to the small amounts available of the labeled granulysin. First the emission spectrum of granulysin labeled with Alexa Fluor 488 together with LUV was measured, which showed an emission peak at 520 nm when excited at 490 nm. Then granulysin labelled with Alexa Fluor 594 was added to the mixture and the emission spectrum was measured again when excited to 490 nm. The result, which shows an emission peak at both 520 nm and 620 nm, reveals a FRET signal when both Alexa Fluor labelled proteins were present (Figure 4A). This indicates an oligomerization of the protein. As a control, Alexa Fluor 488 and Alexa Fluor 594 labelled granulysin were measured together and separately in a buffer solution (0.05 M Tris-HCl, 0.15 M NaCl and 0.001 M $CaCl_2$, pH 7.2). This experiment showed no FRET signal (Figure 4B). From this data we conclude that granulysin is oligomerizing when coming in contact with negatively charged membranes.

The oligomerization of granulysin on phospholipid bilayers was further investigated using confocal laser scanning microscopy to get information about the localization of and time needed for oligomerization. Granulysin labeled with Alexa Fluor 488 was bound to the bilayers and the emission at 520 nm and 620 nm was measured after excitation at 488 nm (Figure 5A, C). Patches of brightly stained granulysin labeled with Alexa Fluor 488 could

seen when exciting with 488 nm and detecting at 520 nm (Figure 5A) but no signal at excitation / emission 594 nm / 620 nm (Figure 5B) or 488 nm / 620 nm (Figure 5C) could be seen as long as no granulysin labeled with Alexa Fluor 594 was added. After adding granulysin labeled with Alexa Fluor 594 to the bilayer with bound granulysin labeled with Alexa Fluor 488 not only the bright patches with excitation / emission 488 nm / 520 nm (Figure 5D) could be seen but also patches at 594 nm /620 nm (Figure 5E). Large patches were not found colocalized. Colocalization for small patches could not be reliably determined as these patches or bright spots are below resolution. When having both labeled granulysin on the bilayer a FRET emission signal with excitation / emission at 488 nm / 620 nm could be detected (Figure 5F). Analyzing the signals (excitation / emission 488 nm / 520 nm and 488 nm / 620 nm) over time after adding granulysin Alexa Fluor 594 an immediate increase of the signal at excitation / emission 488 nm / 620 nm occurred with a concomitant decrease in 488 nm / 520 nm (Figure 5G). The increase of the FRET signal in seconds after adding granulysin Alexa Fluor 594 is an indication for an immediate oligomerization of granulysin after binding to the bilayer. This oligomerization is ongoing with the FRET signal increasing over the whole duration of the experiment.

Oligomerization of Granulysin on Prokaryotic and Eukaryotic Membranes

Granulysin was shown not to permeabilize eukaryotic membranes (Barman et al., 2006) despite the substantial amount that is bound by these membranes. To see if oligomerization is a prerequisite for permeabilization we performed a FRET experiment using vesicles made of *E. coli* and erythrocyte membrane lipid extracts. Respective vesicles were first measured with granulysin labeled with Alexa Fluor 488 followed by granulysin labeled with Alexa Fluor 594 (Figure 6). The results showed a positive FRET peak in the spectrum at 620 nm (excitation at 490 nm) for both prokaryotic and eukaryotic membranes., The FRET signal for granulysin molecules bound to E. coli membranes (Figure 6A) was considerably more pronounced than the FRET signal for granulysin in contact with erythrocyte membranes (Figure 6B) even when initial fluorescent signals at 490 nm / 520 nm were comparable. This implies that granulysin oligomerizes on both prokaryotic and eukaryotic membranes but to a lower extent on eukaryotic membranes. This correlates with the fact that granulysin is not able to induce defects on eukaryotic membranes.

Detection of the Oligomers bound to the Phospholipid Membranes

To further proof stable oligomers formation by granulysin , negatively charged cholesterol containing vesicles (POPG:DPPC:Cholesterol 1:1:1) were incubated for 1 hour together with

1 µM granulysin. The sample was subsequently analyzed by by western blotting under reducing and non reducing conditions. In reducing sample buffer, granulysin is detected as a 9 kDa large protein (Figure 6, lane A), whereas the sample with non-reducing sample buffer showed oligomers of various sizes (Figure 6, lane B). This further proves that granulysin forms stable, detergent insoluble oligomers after binding to a negatively charged membrane.

Discussion

Many small antimicrobial proteins, which bind to the bacterial membrane and kill the bacteria by lysis of the membrane, will not be able to actively penetrate the bacterial membrane unless the concentration of the protein is high enough to form oligomers (Walev et al., 1995). We showed recently that granulysin, one of these small antimicrobial proteins, is able to cause defects on prokaryotic membranes and on negatively charged cholesterol free bilayers mimicking prokaryotic membranes (Barman et al., 2006). Granulysin destroys intracellular bacteria, such as *L. innocua*, after binding to and uptake via lipid rafts by the target cell (Walch et al., 2005). Taken these results together the mechanism of uptake and killing of bacteria by granulysin must fulfill the following criteria: 1. ensure that no premature killing of the infected cell occurs during uptake which would result in release and further spreading of intracellular bacteria. 2. guarantee that granulysin is transferred to and kills the intracellular bacteria.

Granulysin binds in a concentration dependent manner to negatively charged cholesterol containing membranes, typically found in eukaryotic cells (Barman et al., 2006). As we could show is the association of granulysin at high concentrations with negatively membranes of all compositions very fast. The association is slower for lower concentrations of granulysin. Regardless of the starting concentration and the high positive charges on granulysin a substantial amount of granulysin is not irreversibly bound neither by eukaryotic nor prokaryotic membranes. The binding to the cell eukaryotic cell membrane needs to be reversible, for subsequent binding and permeabilization of the bacterial membrane. Using surface plasmon resonance we could clearly demonstrate that a part of the protein remains in solution but also that a part of the protein stays permanently bound to the membrane.

The structurally related protein saposin C has been shown using the atomic force microscope to disturb the organization of the lipids in the bilayers. It binds to the bilayers and progressively recruits additional saposin C molecules to penetrate the membrane, which leads to membrane restructuring (You et al., 2003; You et al., 2004). This correlates with our results, showing a loss of lipids when the bilayers are treated with saposin C as detected in the

biacore. Streptolysin O, which binds to the cholesterol in the membrane (Shany et al., 1974) showed in comparison to granulysin a stronger binding to the cholesterol containing membrane, when after the dissociation period, most of the protein was still bound to the membrane. This suggests that a large fraction of streptolysin O is irreversibly bound to the membrane, which supports the idea of the oligomerization, where the oligomers do not detach from the membrane once bound.

Similar to other cationic proteins of the SAPLIP family, the three dimensional structure of granulysin does not predict pore formation (Anderson et al., 2003). Anderson et al. propose a model for the mechanism of action for granulysin, where each granulysin molecule binds to its neighbouring molecules, applying local forces to a part of the membrane (Anderson et al., 2003). This model correlates with the carpet model, where protein molecules covers the infected cell by first binding to the membrane using electrostatic interactions and, second, permeabilization of the membrane is induced only where the protein concentration is high enough (Anderson et al., 2003; Pouny et al., 1992; Shai, 1999). This is in agreement with our previous results, where we were able to show using the atomic force microscope that granulysin molecules forms areas of defects on the surface of the bilayers (Barman et al., 2006). These defects could originate from oligomerized granulysin molecules, that permeabilized membranes as proposed by Anderson et al. (Anderson et al., 2003). Measuring FRET after incubating labeled granulysin with Alexa Fluor 488 or 594, respectively, to membranes further indicates that the proposed mechanism of binding with subsequent oligomerization leads to permeabilization. The oligomers are formed on negatively charged phospholipid vesicles, as well as on eukaryotic and prokaryotic membranes. On prokaryotic membranes the oligomerization is more efficient than on eukaryotic membranes, which is crucial for granulysin to be able to permeabilize bacterial membranes, but not to harm the target cell membrane.

From this data we conclude that granulysin binds to eukaryotic membranes, mostly in a monomeric form, but can also form oligomers, is endocytosed to reach the compartments with intracellular bacteria. Then the monomeric, reversible bound fraction of granulysin binds to the prokaryotic membranes, oligomerizes on their surface and finally permeabilizes the bacterial membrane leading to lysis of the intracellular bacteria.

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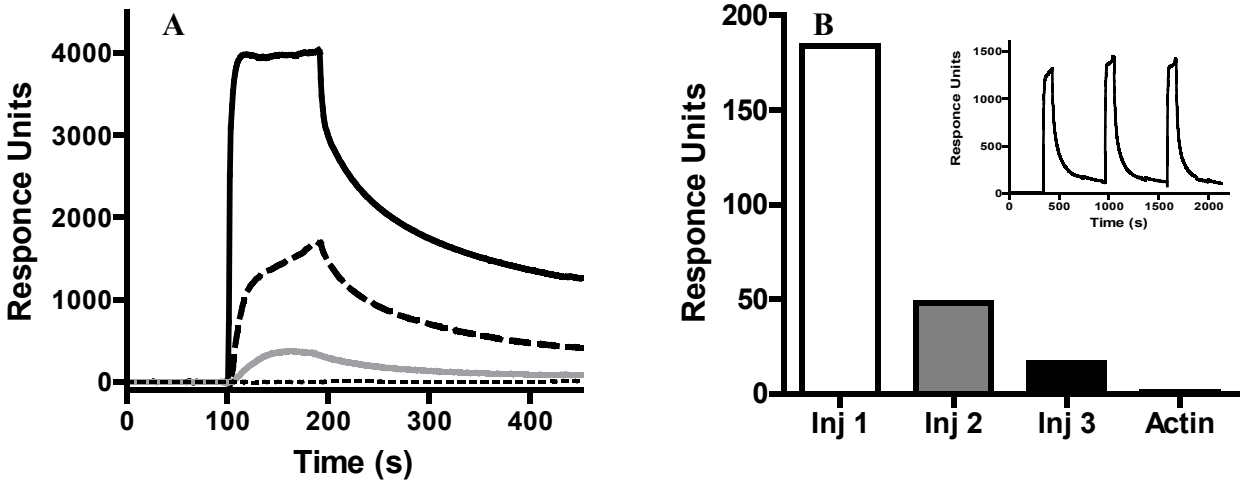

Figure 1. (A) The concentration dependent binding of granulysin to the negatively charged test surface (POPG:DPPC:Cholesterol 1:1:1) in relationship to the uncharged control surface (POPC:DPPC:Cholesterol 1:1:1) is shown by surface plasmon resonance on a L1 chip using a Biacore 3000. The highest concentration of granulysin, 10 μM (solid line) showed the highest binding to the membrane, in comparison to 1 μM granulysin (dashed line), 0.1 μM granulysin (gray line) and the control protein actin-fragment at a concentration of 10 μM (dotted line). (B) The graph shows the sequential binding of 1 μM granulysin added three times to the negatively charged surface (POPG:DPPC:Cholesterol 1:1:1) in relationship to the uncharged control surface (POPC:DPPC:Cholesterol 1:1:1).

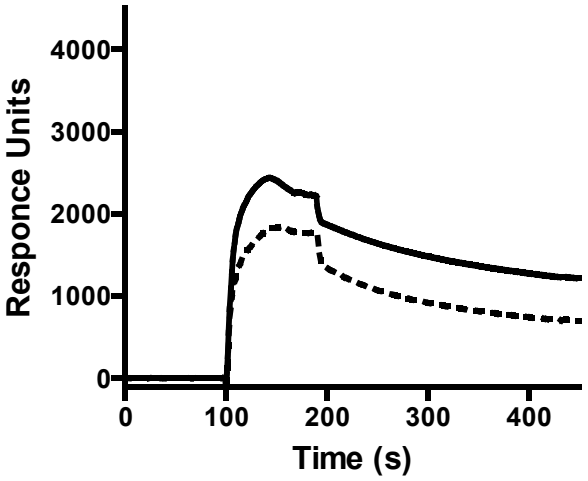

Figure 2. The binding of granulysin to the erythrocyte membranes (dotted line) and *Escherichia coli* membranes (solid line) in relationship to the uncharged phospholipid vesicles (POPC:PDDC:Cholesterol 1:1:1) is shown by surface plasmon resonance on a L1 chip using a Biocore 3000. Granulysin at the concentration of 10 μM is able to bind to both erythrocyte and *E. coli* membranes, with a higher affinity to the bacterial membranes.

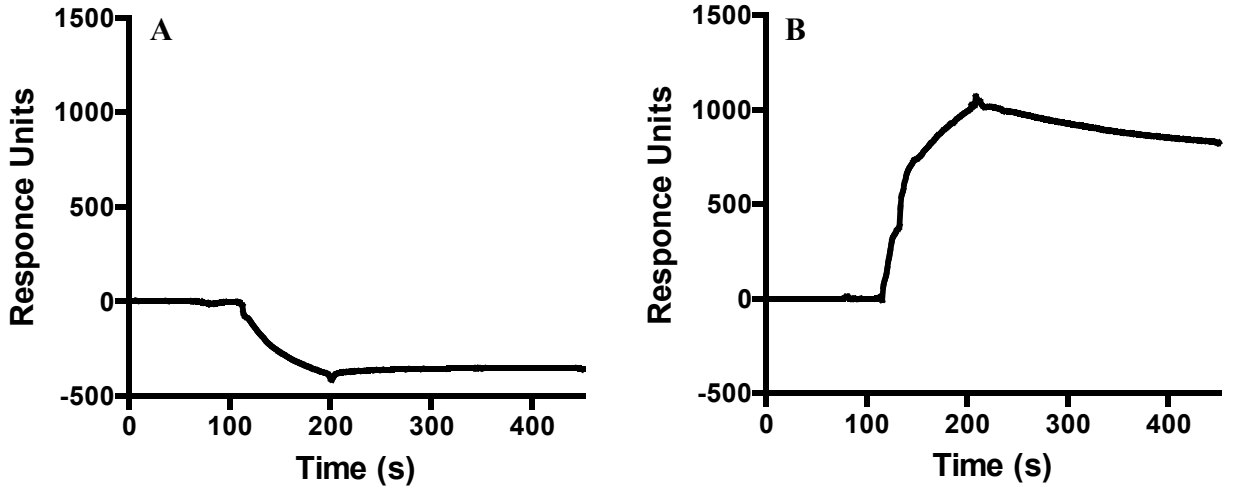

Figure 3. The binding of Saposin C (A) and Streptolysin O (B) to the negatively charged test surface (POPG:DPPC:Cholesterol 1:1:1) in relationship to the control surface (POPC:DPPC:Cholesterol 1:1:1) for Saposin C and (POPG:DPPC 1:2) for Streptolysin O is shown by surface plasmon resonance on a L1 chip using a Biacore 3000. Saposin C (10 μM) clearly removes lipids from the bilayers, which is shown by the negative response in the sensogram, whereas Streptolysin O (2500 U/ml) binds to the cholesterol containing membrane.

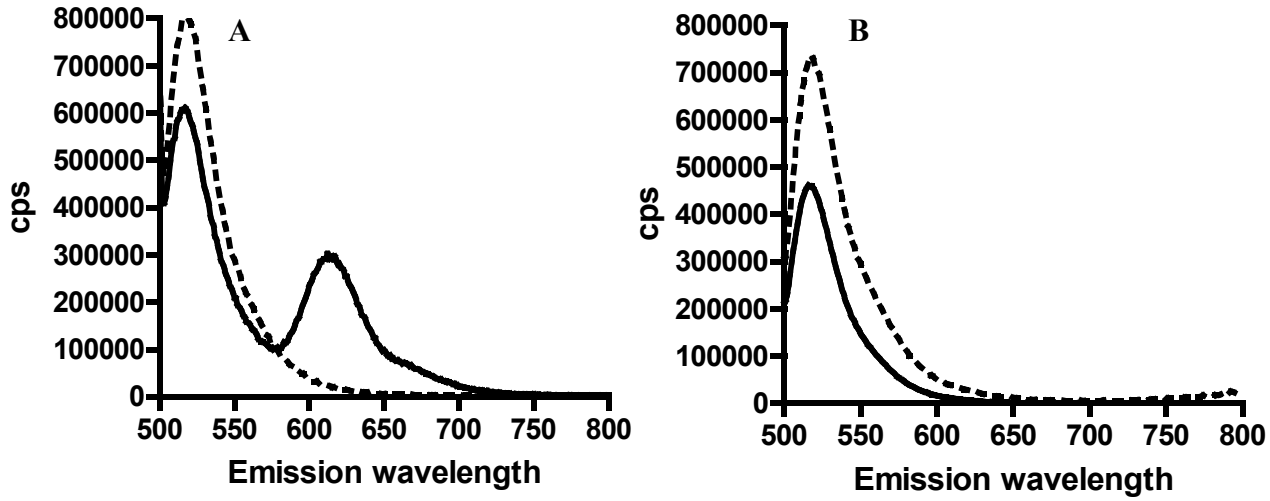

Figure 4. The oligomerization of granulysin was studied using FRET experiments and granulysin molecules labelled with either Alexa Fluor 488 or Alexa Fluor 594. A. The dotted line shows the emission spectrum for granulysin coupled with Alexa Fluor 488 together with negatively charged vesicles (POPG:DPPC:Cholesterol 1:1:1) revealing a peak at 520 nm, and the solid line shows an emission peak at both 520 nm and 620 nm, reveals a FRET signal when both Alexa Fluor labelled proteins were present. B. Shows the same experimental conditions as in A performed in a buffer solution, revealing that granulysin does not oligomererize in absence of a membrane.

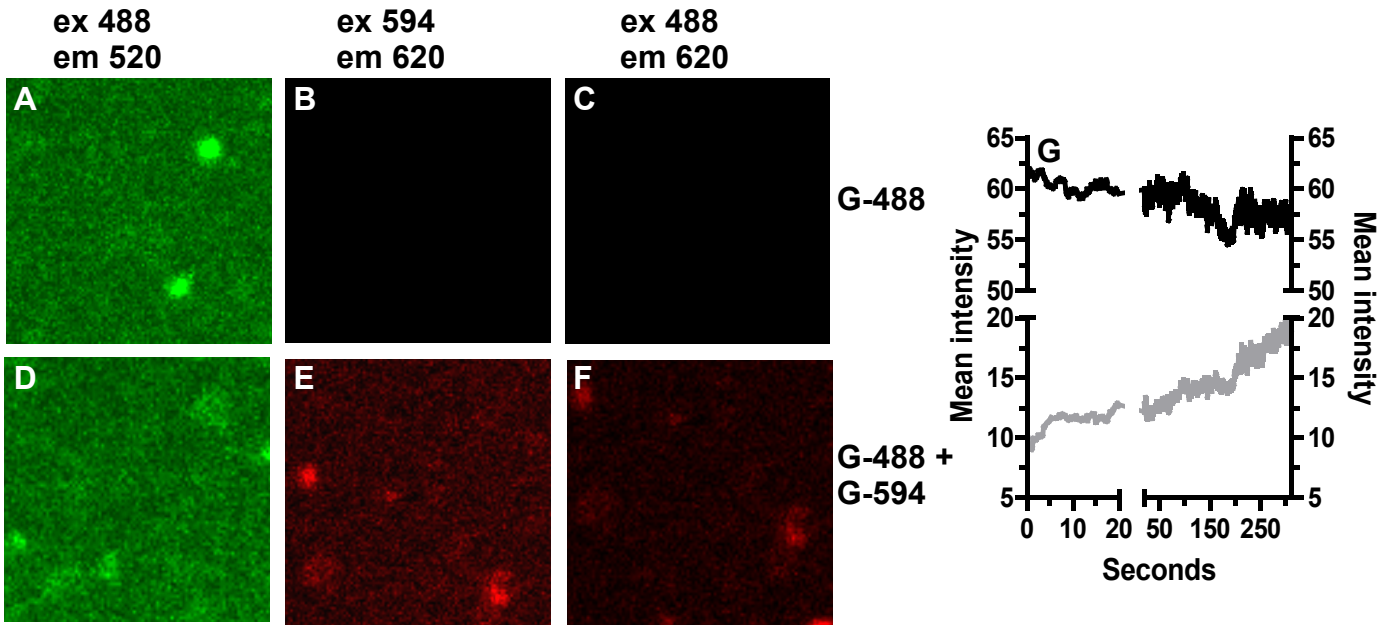

Figure 5. (A) shows the Alexa Fluor 488 coupled granulysin (G-488) bound to the bilayer (λ_{ex}= 490, λ_{em}=520), (B) and (C) shows the same bilayer at the emission wavelenght of 620 nm and the excitation wavelenght at 594 nm and 488 nm, respectively. (D) indicates the signal for G-488 at the timepoint after adding granulysin with AlexaFluor 594 (G-594), wheras (E) and (F) shows the same bilayer at the emission wavelenght of 620 nm and the excitation wavelenght at 594 nm and 488 nm, respectively, indicating the FRET signal in figure (F). The size of the images are 0.5 µm x 0.5 µm. (G) shows the FRET signal of G-594 measured at emission 620 nm after excitation only at 490 nm is shown in gray. The signal is rising over time, revealing that the protein is oligomerizing on the membrane. The black line shows the excitation signal for G-488 recorded at the emission wavelength 520 nm.

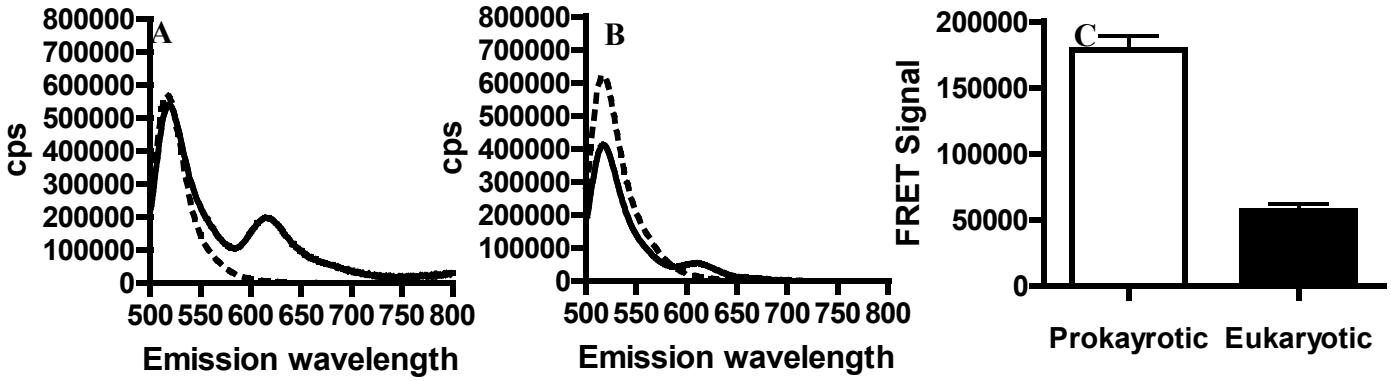

Figure 6. The oligomerization of granulysin on prokaryotic (A) and eukaryotic (B) membranes was studied using FRET experiments and granulysin molecules labelled with either Alexa Fluor 488 or Alexa Fluor 594. The dotted line shows the spectrum for granulysin with Alexa Fluor 488 with a peak at 520 nm, and the solid line shows the FRET signal at 620 nm when both Alexa Fluor labelled proteins are present. The clear difference in the FRET signals of the membrane types is shown in (C), with three times higher signal at the procaryotic membranes, revealing a more extent oligomerization on *E. coli* membranes.

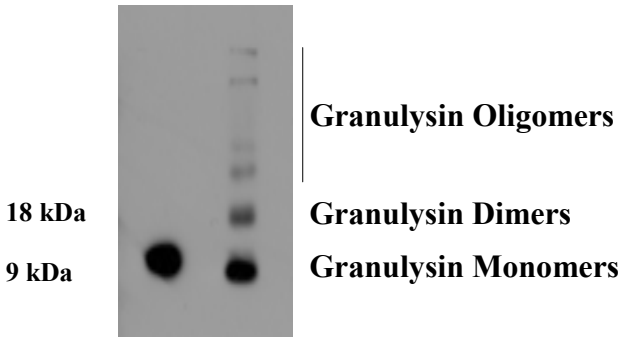

Figure 7. The stable oligomers of granulysin on negatively charged vesicles (POPG:DPPC:Cholesterol 1:1:1) were shown by reducing (left) and non-reducing (right) SDS-PAGE followed by western blotting. The stable oligomers of the non-reduced samples are shown in B.

DISCUSSION

In this study we focused on the membrane binding, activity and oligomerization of the antimicrobial protein granulysin. Membrane active and pore forming toxins, such as amoebapores from the parasite *Entamoeabe histolytica*, are broadly distributed proteins, which form lesions and pores in membranes (Bruhn & Leippe, 1999; Bruhn et al., 2003). Amoebapores, as well as NK-lysin, belong to the same SAPLIP family as granulysin, sharing the particular three dimensional structure with a positively charged surface and their ability to interact with lipids (Bruhn et al., 2003; Sordillo et al., 2005). However, these proteins display differences in their mechanisms of action. All these proteins possess membrane perturbing and antimicrobial activity, which permit the defence against invading pathogens (Hecht et al., 2004; Kaspar et al., 2001). One of the most prominent differences of these proteins is the pH dependency of their activity. NK-lysin is pH-independent, whereas the activity of both granulysin and amoebapores is significantly reduced at pH-values higher than pH 6. Amoebapores are not able to oligomerize at high pH-values (Andra et al., 2007; Winkelmann, Leippe & Bruhn, 2006). This could also be the case for granulysin, which is dependent on the ability to form oligomers for its membrane perturbing activity.

Our finding revealing that granulysin is more active at lower pH values implies that the protein is perfectly adapted for its intracellular role while travelling along the endocytotic pathway (Barman et al., 2006). Granulysin is able to bind reversibly to the infected host cell membrane using the negative charges on the surface, but is only partly able to oligomomerize and therefore cannot permeabilize the eukaryotic membrane (Barman et al., 2006; Sundstrom, In Progress). Instead, granulysin is taken up via the lipid raft mediated endocytotic pathway, where the pH dependency plays an important role for the function and activity of granulysin (Barman et al., 2006; Walch et al., 2005). The pH in the endocytosis compartments changes from 5.9-6.0 in early endosomes to 5.0-6.0 in late endosomes to 5.0 in lysosomes (Geisow &

Evans, 1984; Renswoude et al., 1982), which converts granulysin from lower activity to a highly active protein at low pH in the later stages of endocytosis. This pH dependency of granulysin leads to increased activity and promotes the binding to prokaryotic membranes and killing of intracellular pathogens, such as *L. innocua* and *M. tuberculosis*, which travel along the same route, namely from endosomes via phagosomes to phagolysosomes (Gaillard et al., 1987), where they can be efficiently lysed by granulysin (Walch et al., 2005). Our data also indicate that the protection from lysis of the endosomal compartments may stem from the lipid composition and the cholesterol content of these membranes and, therefore, we suggest this to be part of a mechanism for inhibiting the permeabilization by granulysin (Barman et al., 2006).

To reach the intracellular bacteria, granulysin needs first to bind reversibly to the eukaryotic cell membrane. On the host cell membranes lipid rafts, which are highly concentrated in the regions of the immunological synapse (Xavier et al., 1998), the fraction of granulysin that is tightly bound and oligomerized, applies local stress that might trigger budding and subsequently endocytosis of granulysin-containing membrane vesicles. Subsequently granulysin is able to reach the intracellular bacteria and bind to their membranes. The difference in packing in lipid raft versus non-lipid raft parts of the membrane is due to the saturation of the hydrocarbon chains in raft sphingolipids and phospholipids as compared with the unsaturated state of fatty acids of phospholipids in the liquid-disordered phase. This leads to a highly rigidized membrane structure, with important functions in the uptake and viability of the cell, and also protects the host cell membrane from granulysin mediated lysis (Crane & Tamm, 2004; Simons & Vaz, 2004).

After cholesterol-dependent uptake via lipid rafts, granulysin is transported to early sorting endosomes as indicated by the colocalization with the early endosomal antigen 1, fusing later with phagosomes and lysosomes, and finally lysing the intracellular bacteria. At the same time secreted IFN-γ at locations of infection by the CTLs would enhance the targeting of

granulysin to the *Listeria* containing phagosomes. There is some evidence that IFN-γ triggers upregulation of the small rab5 GTPase in activated macrophages and therefore enhances phagosome maturation (Alvarez-Dominguez 1998; Via 1998). Fusion of granulysin containing early endosomes with phagosomes may be regulated by rab5 and directly correlated with an accelerated maturation of *Listeria* containing phagosomes. This will further increase the efficiency of granulysin mediated killing of intracellular bacteria (Alvarez-Dominguez 1999).

Granulysin is also able to kill bacteria in suspension. Our results of the killing of *Listeria innocua* grown in suspension (Walch et al., 2005) are in agreement with data obtained in other workgroups using a variety of microbial pathogens including *Listeria monocytogenes* (Stenger, 1998, Ernst 2000). The binding of granulysin to bacterial membranes is shown here for the first time (Walch et al., 2005) and is explained by the multitude and the distribution of positive charges of granulysin (net charge +11 at neutral pH), and on the other hand, the composition of the prokaryotic and eukaryotic membranes containing acidic phospholipids, such as phosphatidylglycerol and cardiolipin in prokaryotic membranes as well as additional cholesterol in eukaryotic membranes (Gidalevitz et al., 2003; Kurz et al., 2005; Pomorski et al., 2004). The positive charges from the arginine residues of the granulysin molecule attract the protein to the negative charges in the membrane phosphates.

The charges on the surface of granulysin are essential for the binding to both the eukaryotic and to the prokaryotic membrane, and the fact that eukaryotic membranes contain cholesterol (Albert & Boesze-Battaglia, 2005; Ohvo-Rekila et al., 2002; Slotte, 1999) does not inhibit the binding of the protein, indicating that granulysin is able to effectively bind to both the host cell membrane and the bacterial membrane, which is of importance for an effective lysis of intracellular bacteria. Granulysin binds to and permeabilizes prokaryotic membranes in a concentration dependent manner, which suggests a cooperative action of the granulysin (Barman et al., 2006; Sundstrom, In Progress). This cooperative action of granulysin has also

been suggested by Anderson et al. (2003), who describe that many granulysin molecules may come together to form the membrane lesions, which leads to a scissoring motion of granulysin to further expose the lytic surface of the molecule on the prokaryotic membrane (Anderson et al., 2003).

Other positively charged toxins are defensins, which also belong to the family of pore forming toxins. They are secreted by activated neutrophils, and their mechanism of action correlates to granulysin. These small cationic toxic peptides, which after release from the granules of neutrophils, bind to the target cell membrane in a charge dependent manner, but in contrast to granulysin, defensins induces an ion flux by pore formation (Sahl et al., 2005). These peptides also show a great difference in the mechanism of action against prokaryotic and eukaryotic membranes, which is thought to be due to the greater capacity of the eukaryotic cells for membrane repair (Bals & Hiemstra, 2004; Lichtenstein et al., 1988), but as shown in our results, the mechanism for the defence against antimicrobial proteins of the eukaryotic cell membrane could be due to the differences in the eukaryotic and prokaryotic membranes.

In general, the resistance of eukaryotic membranes against membrane active and pore forming proteins can be due to the absence of a high affinity binding site, the capacity to repair lesions and the inhibition of the insertion of the pore forming domains despite the assembly of oligomers (Valeva et al., 2000). We clearly showed that cholesterol is the component preventing the permeabilization and partly the oligomerization of granulysin on membranes, due to the tighter membrane packing and rigidity of the lipid rafts as well as the non lipid raft part of the membrane, and therefore works as a protector for the antimicrobial effect against eukaryotic cells. Prokaryotic membranes do not contain cholesterol and allow oligomerization of granulysin in a higher extent than on eukaryotic membranes, and are therefore lysed by the granulysin oligomers. Also other membrane active proteins, such as listeriolysin O, have shown to oligomerize to perform its action intracellularly. The enhanced activity of

listeriolysin O at low pH and low cholesterol concentrations reveals a toxin completely adapted for its intracellular role in mediating escape of bacteria from the phagosomes (Bavdek et al., 2007).

The mechanism of action of granulysin differs from the other lytic proteins released from the acidic granules of CTLs and NK cells. The active form of perforin also binds in a monomeric form to the cell membrane, but not charge dependently, instead using the phosphatidylcholine as a receptor for binding. After the binding, also perforin forms oligomers on the membrane, but instead of lipid raft dependent endocytosis, perforin forms pores on the target cell membrane, where granzymes and granulysin can enter (Keefe et al., 2005; Tschopp et al., 1989). The perforin induced cell death is a result of osmotic lysis or the influx of Ca^{2+} ions (Liu et al., 1995a; Liu et al., 1989). Granzyme B on the other hand is able to bind to the target cell membrane using charges, but it cannot induce target cell death without the presence of perforin, because it is needed for the intracellular delivery of granzyme B (Giesubel et al., 2006; Kurschus et al., 2004). In contrast, granulysin can enter the target cell and lyse intracellular bacteria without the presence of perforin (Walch et al., 2005).

The entering of granulysin through the eukaryotic membrane can be explained by the reversible binding of granulysin on the host cell membrane. Granulysin oligomerizes on negatively charged membranes, and on eukaryotic and prokaryotic membranes, but in a lower extent on eukaryotic membranes, which indicates the reversible binding to eukaryotic membranes where only a minor fraction is oligomerized, and the monomeric fraction of the protein continues through the endocytotic pathway towards the intracellular bacteria, binds and oligomerizes on their surface, and finally permeabilizes the bacterial membrane, leading to lysis. This finding is of importance for understanding how granulysin binds to the host cell membrane, is endocytosed and then is capable to bind again to the bacterial membrane, leading to lysis of the intracellular bacteria.

Also other toxins, such as Staphylococcus α enterotoxin, are secreted and bind to the target membrane in a monomeric form. First oligomeric pre-pores are formed, and only when the pore forming sequence of the toxin is inserted into the bilayers a pore can be formed. The oligomeriozation of this toxin is allowed on lipid rafts, whereas the fraction of the toxin that is bound outside of this microdomain in a monomeric form are only transiently bound to the membrane, correlating to the membrane binding activity of granulysin on eukaryotic membranes (Valeva et al., 2006).

This study provides important information for understanding the function, activity and the mechanism of action of granulysin and other positively charged antimicrobial proteins. We give an insight how they interact with eukaryotic versus prokaryotic membranes and also an explanation why eukaryotic membranes are protected from being lysed by granulysin as well as the mechanism how granulysin is able to reach and kill intracellular bacteria.

CONCLUSIONS

Based on these results we conclude that granulysin binds transiently to the infected host cell membrane using electrostatic interactions, but is able to only partly oligomerize on these membranes. The transiently bound fraction of granulysin enters the cell via lipid raft mediated endocytosis and is transferred via early and late endosomes and then fusing with bacteria-harbouring phagosomes and lysosomes, the compartments were intracellular bacteria are located.

The pH value in the endocytotic compartments gets gradually more acidic, leading to a higher activity of granulysin due to the more positively charged surface. Granulysin binds then to the negatively charged bacterial membrane, oligomerizes to be able to add local forces on the membrane and to finally permeabilize the bacteria leading to lysis.

The cholesterol content of the host cell membrane plays a particularly important role in the potential of granulysin to perturb the membrane. Not only lipid rafts but also non-raft parts of the eukaryotic membrane contain cholesterol and are therefore not permeabilized. This seems to be a protective property of the eukaryotic membrane. The infected host cell takes up granulysin through the cholesterol enriched lipid rafts and transports the lytic protein to the intracellular bacteria. This mechanism is illustrated in Figure 3.

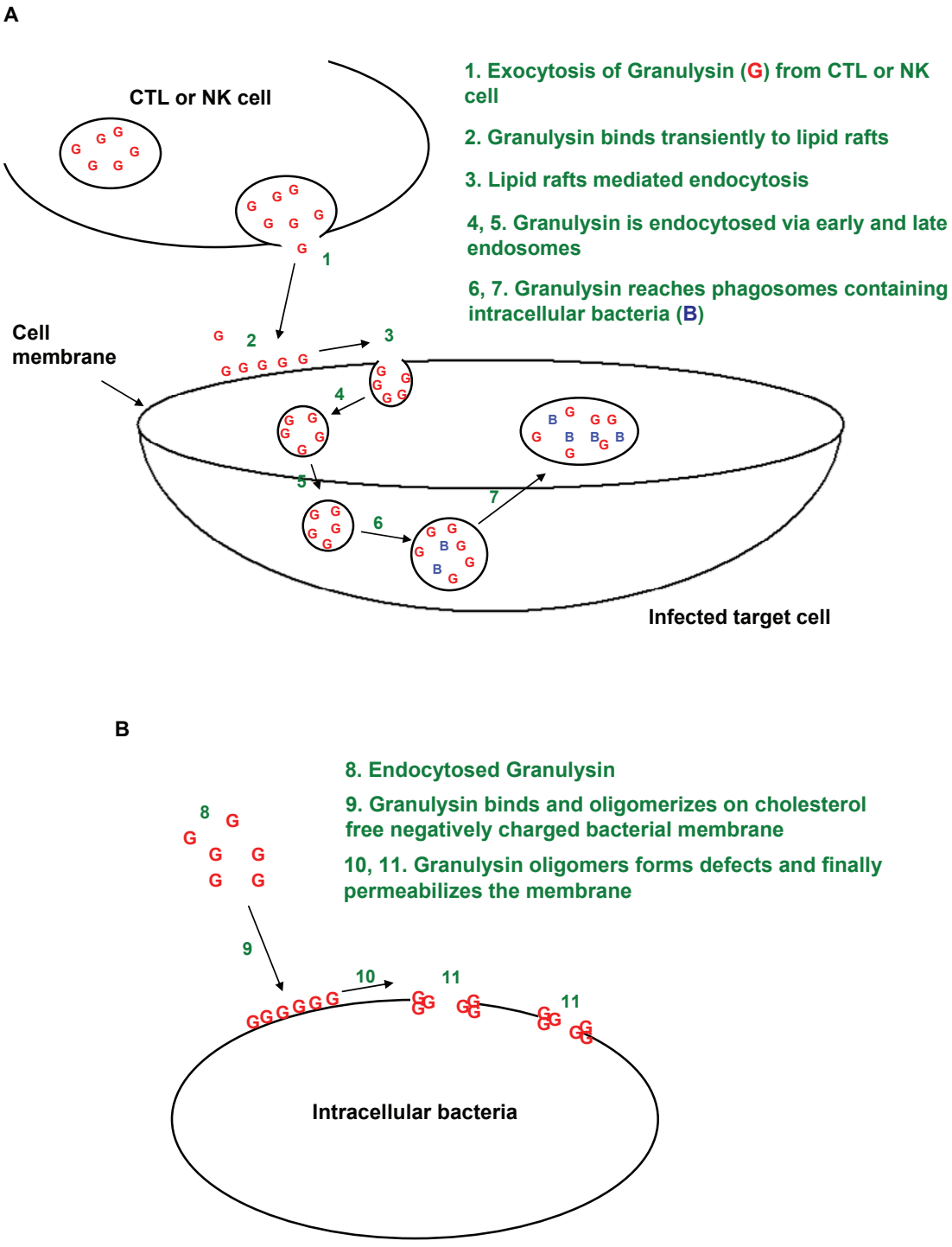

Figure 3. The mechanism of granulysin-mediated killing. (A) Granulysin molecules are released during granule exocytosis from activated CTLs or NK cells (1). Granulysin binds to the lipid rafts on the infected target cell membrane (2) and is endocytosed via early and late endosomes

(4,5) to phagosomes and lysosomes (6,7). (B) The pH value is more acidic in the later compartments of endocytosis, leading to an increased binding of the active endocytosed granulysin (8) to the intracellular bacterial membranes (9). Granulysin oligomerizes (10) and permeabilizes the cholesterol free bacterial membrane leading to lysis and killing of the bacteria (11).

FURTHER STUDIES

To continue with the study of how granulysin interacts and permeabilizes membranes, circular dichroism (CD) could be used to investigate the conformational changes of the protein when it binds to eukaryotic versus prokaryotic membranes. Since the oligomers of the granulysin molecules could only bee shown by FRET experiments, due to the resolution limitations of the AFM, the CD technique would give valuable information about the differences in binding and acvtivity of granulysin on different membranes.

The binding and activity of the protein could also be tested on membranes extracted from different bacteria, e.g *Chlamydia pneumoniae* and *Mycobacterium tuberculosis*.

Finally, a computer simulated model showing how the protein interacts with and binds to membranes could be completed.

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